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Metabolic Imaging of the Organ of Corti – A Window on Cochlea Bioenergetics

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Abstract

Hair cell loss is a major cause of sensorineural hearing loss. We have developed a method to examine metabolic events in hair cells in response to stimuli known to cause hair cell loss, such as acoustic trauma and aminoglycoside administration. The method employs two-photon excitation of the metabolic intermediate, reduced nicotinamide adenine dinucleotide (NADH), in hair cell mitochondria in an explanted mouse cochlea. Using this method, we show evidence that the aminoglycoside gentamicin selectively affects the level of mitochondrial NADH in outer hair cells, but not inner hair cells, within minutes of administration.

Keywords

NADH; multi-photon confocal microscopy; aminoglycoside; gentamicin; sensorineural hearing loss

1. Introduction

It is a common theme of auditory neuroscience that cochlea outer hair cells (OHCs) are substantially more vulnerable to insult than inner hair cells (IHCs), whether the insult is from excessive noise, aminoglycoside ototoxicity, or presbycusis (Hamernik et al., 1984; Hunter-Duvar and Bredberg, 1974; Saunders et al., 1985; Tarnowski et al., 1991). Furthermore, basal turn OHCs (from the organ of Corti in the region of high best frequency) are more vulnerable than more apically-located OHCs to the same insults. The common basis for OHC loss by all three etiologies appears to be apoptosis triggered by the generation of reactive oxygen species (ROS) (Cotanche, 2008), or, in presbycusis, by a diminished capacity to remove ROS (discussed in Ohlemiller and Frisina, 2008). Indeed, numerous attempts have been made, and continue to be made, to ameliorate OHC loss in animal models by blocking apoptotic signal transduction pathways or by scavenging free radicals, with varying degrees of success (Campbell et al., 2007; Endo et al., 2005; Hamernik et al., 1984; Ylikoski et al., 2002). However, little effort has been made to understand the physiological or biochemical bases of these observations.

The importance of ROS and apoptosis in OHC death point to a possible metabolic etiology. One major apoptotic trigger is free radicals generated by mitochondrial enzymes (Lenaz,

1998). This would suggest that OHCs operate at an enhanced metabolic rate, or have diminished ability to cope with excess ROS. However, there are no known energetic processes such as ionic pumps present in OHCs to account for the differences in susceptibility that would not also be present in IHCs. The one major difference between OHCs and IHCs, the activity of the motor protein prestin, does not seem to be an ATP dependent process (Ashmore, 2008).

Currently, confocal microscopy is being applied to improve understanding of metabolic events in several tissues, including neurons, cardiac muscle, cancer and precancerous tissue, and to cultured cells (Blinova et al., 2004; Brandes and Bers, 1996; Mayevsky and Rogatsky, 2006; Vishwasrao et al., 2005). These studies take advantage of the fact that the reduced form of the important metabolic intermediate nicotinamide adenine dinucleotide (NADH) is intrinsically fluorescent whereas the oxidized form (NAD⁺) is not. NADH may be excited by ultra-violet (UV) light or by two photons of near infra-red (NIR) light. Thus changes in NADH fluorescence may be taken to give a measure of changes in metabolic processes in living cells.

In this study, we have applied multi-photon microscopy to the study of NADH fluorescence in hair cells in a living explanted mouse cochlea preparation. The preparation resembles one described in an earlier study (Tiede et al., 2007), but has undergone considerably less dissection (see Methods). In the previous study, we showed that percent of NADH decreased over time, presumably as metabolic reserves were consumed. We observed that this decrease in NADH occurred more rapidly in OHCs than in IHCs. Given that NADH levels were not constant, this preparation was not suitable for comparative studies. With our new preparation, we have achieved stable levels of NADH fluorescence for up to one hour. Thus this preparation is now suitable for use in testing the effects of traumatic stimuli on hair cell metabolism. As our first study, we have investigated the effects of an aminoglycoside antibiotic, gentamicin, on NADH fluorescence in the organ of Corti. In order to assess the uptake of aminoglycoside into hair cells, we mixed unlabeled gentamicin with a Texas Red-coupled gentamicin (GTTR) developed by one of us (Dai et al., 2006).

2. Results

Explant Viability

NADH imaging apical and middle turns of explant preparations showed that NADH fluorescence levels in all cell types remained stable (within error) over the course of 60 minutes (data not shown). For further determination of the viability of the explant, preparations were incubated in solution containing calcein-AM and propidium iodide (PI) (see Methods). PI labeled the nuclei of cells without intact membranes while calcein fluorescence indicated active esterases in the cytoplasm. As such, live undamaged cells would therefore be expected to exhibit calcein fluorescence but not PI fluorescence (Nichols et al., 2005). Staining with these two indicators showed that 85% of OHCs and 95% of IHCs were viable in this preparation, even after 60 minutes (Fig. 1).

Mitochondrial Localization of NADH Fluorescence

The fluorescence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) is indistinguishable from that of NADH. However, NADPH is a cofactor in anabolic processes and enzymes using NADPH as a co-factor are also found in mitochondria. In our previous study in a more extensively modified explanted cochlea preparation (Tiede et al., 2007), we demonstrated that the fluorescence probably originated from NADH, rather than NADPH, by showing that the fluorescence could be manipulated by the metabolic poison sodium cyanide and the metabolic uncoupler carbonylcyanide-4-(trifluoromethoxy)-

phenylhydrazone (FCCP), agents that specifically affect mitochondrial oxidative metabolism. We repeated those studies in this preparation and again found that the fluorescence originated from mitochondria, and therefore most likely from NADH only (data not shown).

Effects of Gentamicin Uptake

As indicated by GTTR fluorescence, gentamicin was taken up into hair cells and pillar cells rapidly. Fig. 2 shows the progressive increase in GTTR fluorescence as a function of exposure time and preparation depth. At the first time point examined ($t = 0$, i.e., immediately after application of the GTTR-containing solution), GTTR fluorescence was observed associated with the apices of both hair cell types, and over the course of 30 minutes rose to fill the entire cell volume. Analysis of GTTR fluorescence indicated that all cell types took up gentamicin without statistically significant differences in volume concentrations (Fig. 3A). However, the effect of gentamicin on NADH fluorescence in OHCs was significantly different from that in IHCs or pillar cells. As indicated in Fig. 3B, levels of NADH fluorescence in OHCs dropped significantly over the course of a half hour, beginning at the 10 minute time point, while levels of NADH fluorescence were not significantly different from baseline levels in IHCs or pillar cells. The NADH fluorescence decrease in the OHCs seemed to reach a stable level at 91% of the initial value within 15 minutes of exposure to gentamicin.

3. Discussion

Explant Viability

The results of the NADH stability study show great improvement in preparation longevity when compared to our previously reported data (Tiede et al., 2007). This finding should not be surprising as the method brings the organ of Corti closer to biological temperature, at which it is expected that enzyme function is optimal for maintaining homeostasis. The traditional practice of using chilled media when removing the organ of Corti may be preferred for fixed tissue assays, because it would be expected to slow or even halt cellular processes, thus permitting more accurate capture of the state of the preparation at a particular time. However, for live tissue imaging, it is vital to maintain the natural working environment as much as possible allowing for the closest approximation to the function of the organ of Corti in the living animal. It is salutary, therefore, to consider that most explanted cochlea preparations are studied at room temperature (Richter et al., 1998; Scherer and Gummer, 2004; Ulfendahl et al., 2002).

Calcein and PI fluorescence indicated that our preparation was viable for time periods of up to one hour. We were unable to obtain similar results using the hemicochlea preparation (Teudt and Richter, 2007), and as such have determined that our preparation is to be preferred for the type of live cell studies used in our laboratory.

Gentamicin Assay

It has long been known that OHCs experience greater rates of cell death than IHCs consequent to exposure to aminoglycosides, including gentamicin. This finding leads us to suggest that the cellular responses of OHCs and IHCs to aminoglycosides are different in some crucial way. For the first time, we are clearly able to observe differences in cellular metabolism occurring during aminoglycosides exposure well before cell death has begun to set in. The significant drop in NADH fluorescence is indicative of perturbation of the OHC mitochondrial metabolism. This could be the result of changes or dysfunction in metabolism of the OHC or due to early events in mitochondrial mediated apoptosis. Given that the average fluorescence intensities of GTTR label in each cell type were similar, our findings

point to a fundamental difference in the way in which OHCs process aminoglycosides. These data also show that gentamicin is potentially fatal to murine OHCs in vitro. In vivo, murine hair cells are pharmacologically sensitive to most aminoglycoside antibiotics administered at very high concentrations compared to other rodents (rats, guinea pigs) and humans. However, gentamicin causes systemic toxicity and death prior to the onset of ototoxicity (Wu et al., 2001). Murine cochlear hair cells are equally as sensitive to acute aminoglycoside administration in vitro as guinea pig hair cells (Dehne et al., 2002; Dulon et al., 1989; Gale et al., 2001; Richardson and Russell, 1991). Our findings here show that murine OHCs in situ are more sensitive to gentamicin than IHCs, and that these concentrations are less than or similar to other aminoglycosides used (1-2 mM) to induce OHC toxicity in in vitro preparations.

4. Experimental Procedure

Explant Preparation

Post-natal day 28-31 FVB mice were sacrificed by CO₂ inhalation followed by decapitation. Both cochleas were removed from the skull and placed in warmed (37°C) L-15 media (Sigma) and vestibular bones were left attached. A small opening was made in the bone over scala vestibuli of the bottom third of the middle turn of one cochlea. The preparation was mounted on a special stage adapter for proper position and immersed in imaging buffer (see below). When correctly positioned, the organ of Corti of this location could be visualized from above. The bone of the cochlea wall under this location is sufficiently translucent that a differential interference contrast image could be formed of the organ of Corti in a plane parallel to the basilar membrane, which aided localization prior to fluorescence imaging. Hair cells and supporting cells were readily recognized and differentiated. The preparation was oriented as close to parallel as possible to the basilar membrane plane. All animal care and handling was in accordance with an approved Creighton University Institutional Animal Care and Use Committee protocol.

Imaging Methods

For imaging of NADH, the organ of Corti was excited using femtosecond pulses of near-infrared illumination (740 nm) from a tunable Chameleon XR laser (Coherent Inc., Santa Clara, CA) by scanning across the sample with an LSM510 laser scanning microscope using a 40x, 0.8 NA water immersion objective (Carl Zeiss Inc., Thornwood, NY). The region of interest was located below the tectorial membrane, and required an average laser power of 30.0 ± 0.5 mW to excite intrinsic fluorophores. Successive focal planes were imaged in 2.5 μ m steps for a total depth of 55-60 μ m, more than sufficient to encompass the entire depth of OHCs, IHCs and pillar cells at this location close to the basal turn. The interval between planes was chosen for optimum reconstruction of the cell volume without gaps. Images of 512x512 pixels were acquired at approximately 4/s with a pixel dwell time of 1.6 μ s.

NADH fluorescence emission was selected using a 500 nm long pass dichroic mirror (500 DCXR, Chroma Technology, Brattleboro, VT) and detected by a photomultiplier tube with a 460/80 band pass filter (Chroma Technology, Brattleboro, VT). Previously we have shown that the blue fluorescence isolated by the 460/80 band pass filter is spectrally consistent with NADH, and that responses to cyanide and FCCP indicate that this NADH fluorescence is mitochondrial in nature (Tiede et al., 2007). Four scans were acquired and averaged to produce a single image.

For imaging with calcein and PI, each preparation was incubated in 50 μ M calcein (Invitrogen) and 50 μ g/ml PI (Invitrogen) in Liebovitz's L-15 medium (37°C) for 15 minutes at room temperature before washing with an imaging medium consisting of 135

mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 20 mM HEPES, 5 mM glucose, and 10% Bovine Serum Albumin (Sigma). For imaging of calcein and PI, we used 488 nm excitation light from an argon ion laser. The fluorescence was passed through the Zeiss 488 nm blocking filter, de-scanned through a one Airy unit pinhole and calcein fluorescence reflected off of a 545 nm dichroic mirror before being passed through a 500-550 nm band pass filter in front of the photomultiplier. PI fluorescence was passed through the 545 nm dichroic mirror, de-scanned through a one Airy unit pinhole, and filtered by a 560 nm long pass filter before reaching the photomultiplier. For imaging of GTTR, the imaging solution contained 300 µg/ml gentamicin and 1 µg/ml GTTR (Dai et al., 2006). GTTR was excited using a 543 nm HeNe laser. The fluorescence was passed through a 543 nm blocking filter, de-scanned through a one Airy unit pinhole and passed through a 545 nm dichroic mirror and a 565-615 nm band pass filter before reaching the photomultiplier. For all GTTR experiments, scans of the preparation were alternated between NADH and GTTR for each focal plane in the series.

Image Analysis

Images were analyzed by selecting individual cells as regions of interest in each focal plane, and then the regions corresponding to a single cell were summed to obtain a total value for the cell. The value obtained was divided by the total number of pixels in all ROIs encompassing a cell to determine an average pixel value that was independent of cell dimensions. Standard errors on the mean were calculated based on the number of cells used to obtain the average. For the first image, the error bars were estimated using the variation in the un-normalized data and propagated using standard error analysis (Bevington, 1969).

For calcein and PI stained images, the image stacks were compressed and the number of calcein and PI labeled cells was counted. The percentage of viable cells was determined by dividing the number of calcein labeled cells by the total number of cells (the sum of the calcein labeled and PI labeled cells).

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Figure 1.

Example of the labeling observed after 60 minutes in a viability study of the explant preparation. Image prepared as a collapsed z-stack. Calcein fluorescence in green, PI fluorescence in red. Green cells are viable, red cells are not.

Figure 2.

Images of gentamicin treated organ of Corti. NADH images are pseudo-color blue (A-D), and GTTR images are pseudo-color red (E-H). Two separate focal planes are shown: one at the top of the preparation (A,B,E,F) and one 30 microns below the surface (C,D,G,H). Each focal plane is shown for two time points 1 minute after addition of gentamicin, (A,C,E,G) and after 30 minutes of incubation (B,D,F,H). Note that gentamicin labels the stereocilia of the OHCs first before diffusing down through the cell at the later time periods. NADH fluorescence drops of visibly in the OHCs during the 30 minutes of incubation.

Figure 3.

A) GTTR fluorescence concentration as a function of time. Results are normalized to the initial values. Uncertainties represent the standard error of the mean for 4 animals. There was no statistical difference in the concentrations between cell types at any time point. B) Normalized NADH fluorescence in OHCs, IHCs, and pillar cells as a function of time following incubation with gentamicin. Results are normalized to the initial intensity values. Uncertainties represent the standard error of the mean for 4 animals. * indicates significantly different from initial baseline ($P < 0.05$).