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Role of STAT1 and Oxidative Stress in Gentamicin-Induced Hair Cell Death in Organ of Corti

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Abstract

Rationale—Oxidative stress plays a critical role in gentamicin-induced hair cell death. Prior work has implicated the cytoplasmic transcription factor STAT1 as a potential mediator of drug-induced ototoxicity, but role in aminoglycosides is largely unknown. This study investigated aminoglycosides-induced cell death, exploring contributions of reactive oxygen species and STAT1 pathway in injury and protection.

Methods—Neonatal murine organ of Corti explants from 2-3 day postnatal pups (n=96) were treated with gentamicin at (4 μ M, 50 μ M) for 4-72 hours, with/without protectants. Effects on STAT1 pathway and gentamicin-induced hair cell death were measured with 50 μ M Epigallocatechin Gallate (EGCG, a STAT1 inhibitor) and all-trans retinoic acid (atRA, a STAT1 activator). Hair cell morphology was evaluated and hair cell loss was quantified with cytochrome c oxidase (COX) staining. Mitochondrial membrane potential was assayed and superoxide generation and suppression was measured with dihydroethidium (DHE) staining.

Results—Co-administration of 50 μ M EGCG conferred protection from 4 μ M gentamicin toxicity ($p < 0.001$), whereas atRA potentiated gentamicin-induced hair cell death ($p < 0.001$). On immunohistochemistry, STAT1 phosphorylation at threonine 727 (Ser⁷²⁷) residues was increased at 72 hours with 4 μ M gentamicin. With administration of 50 μ M gentamicin, there was activation of STAT1 Tyr⁷⁰¹ at 4 hours and STAT1 Ser⁷²⁷ at 16 hours. Gentamicin dissipated mitochondrial membrane potentials, and EGCG attenuated gentamicin-induced oxidative stress at 72 hours.

Conclusion—EGCG protected outer hair cells from gentamicin toxicity in a cochlear explant model, with the underlying mechanism involving both reactive oxygen species (ROS) suppression and STAT1 inhibition.

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Introduction

Hearing loss and vestibular impairment due to inner ear sensory hair cell death are major side effects of aminoglycoside antibiotics. Although the aminoglycosides demonstrate ototoxicity and nephrotoxicity, their low cost of production, widespread availability, and high efficacy leads to significant ongoing consumption in the United States and globally. They remain first-line therapy for neonatal sepsis and multi-drug resistant tuberculosis. Oxidative stress has been implicated in inner ear injury, and potential sources and targets for cellular reactive oxygen species (ROS) are still poorly understood, introducing challenges in finding effective preventative and therapeutic options. Attenuation of ROS and/or downstream pathways may provide opportunities for targeted protection against this form of hearing loss.¹

One approach to understanding the mechanism of aminoglycoside injury to the inner ear and its prevention is through comparison to other pharmacological insults, such as the ototoxicity arising from cisplatin. Aminoglycosides and cisplatin both induce a stereotypical pattern of injury to the inner ear, which involves a base-to-apex progression of outer hair cell loss along the organ of Corti, resulting in preferential high frequency hearing loss. Vestibulotoxicity and nephrotoxicity are also commonly observed with these drugs. ROS production, a manifestation of cellular stress, occurs after aminoglycosides and cisplatin therapy, as well as from noise and aging. Despite such similarities, differences in mechanism have been noted for ototoxic agents for potential otoprotectants.^{2,3}

Prior work has implicated Signal Transducer and Activator of Transcription 1 (STAT1) as a potential mediator of cisplatin-induced ototoxicity,⁴ but the role of STAT1 in aminoglycoside ototoxicity remains largely unknown. Epigallocatechin gallate (EGCG), a green tea extract, is a potent antioxidant and STAT1 inhibitor. It has not previously been studied in relation to aminoglycoside ototoxicity and was explored as a putative protectant and to better understand prevention of injury. All-trans Retinoic Acid (atRA) can be administered to upregulate STAT1 because the murine STAT1 promoter possesses a motif that functions as a retinoic acid response element.⁵ We examined the role of ROS formation and assayed for STAT1 suppression or activation and its relation to gentamicin ototoxicity. EGCG, atRA, and gentamicin were used in varying combinations to investigate the possible contribution of STAT1 activation and oxidative stress to gentamicin-induced hair cell death.

Materials & Methods

Animals

Mature CBA/J mice obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) were used for breeding. The animals were maintained on a 12-hour light/12-hour dark schedule with a regular diet and free access to water. Male and female mice were housed together after a one-week acclimation period. Procedures were performed in accordance with the animal protocol # PRO00005637 under the primary investigator (MJB). All experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA) and University of Michigan's Unit for Laboratory Animal Medicine (ULAM).

Explant Preparation

Post-natal 2-3 day mice were humanely euthanized and temporal bones dissected. Cochleae were extracted and immersed in cold Hank's Balanced Salt Solution, and lateral wall tissues and auditory nerve bundle were stripped away leaving the organ of Corti and spiral ganglion cells. A culture dish was prepared by polymerizing 15 μ L of rat tail collagen solution for 15 minutes on a 35-mm dish. One mL of culture medium was added, consisting of Basal Medium Eagle, 1% serum-free supplement (Gibco, Grand Island, New York), 1% bovine serum albumin, 5 mg/mL glucose, and 10 U/mL penicillin G. Cochlear explants were then incubated for 4 h (at 37°C, 5% CO₂) after which an additional 1.5 mL of culture medium was added to submerge explants.

Experimental Design Synopsis

In the chronic injury paradigm, 4 μ M gentamicin (Sigma-Aldrich, Saint Louis, MO) is administered with or without protectant for a 72 hour period to model a base to apex gradient of hair cell loss, in which approximately one half of hair cells are lost preferentially in the hook, base, and middle turn of the organ of Corti. A 50 μ M gentamicin treatment regimen (acute injury paradigm) was used to achieve maximal induction of STAT1 phosphorylation at early time points (e.g. 4 to 24 hours), to elucidate potential mechanisms of injury. The experiments compared negative control (untreated) explants to those treated with gentamicin, EGCG (Sigma-Aldrich, Saint Louis) or atRA (Sigma-Aldrich, Saint Louis, MO) alone, and Gentamicin plus either EGCG or atRA. Therefore, varying combinations of reagents, dosing, and time points were used for study of several permutations, with a total of 96 explants assigned to the experimental conditions. Separate preparations were required for quantification of hair cells, immunostaining for STAT1 activation, assessment of mitochondrial membrane potential, and DHE assays.

Treatment of Explants

In all cases, explants were incubated for 2 days at 37°C, 5% CO₂ to allow recovery from dissection stress before being exposed to treatment conditions. The initial medium was exchanged with new media. After this incubation, the explants were then either administered low-dose (4 μ M) gentamicin, high-dose (50 μ M) gentamicin, or no additional treatment. Once treatment with gentamicin was initiated, the same gentamicin concentration was maintained in media until the end of the experiment. For assays of protection from/potential of gentamicin injury, pre-treatment occurred 1 hour prior to gentamicin administration with EGCG (50 μ M) or atRA (10 μ M or 100 μ M). Vehicle control incubations were carried out in 0.2% DMSO to control for any effects of solvent. For explants in the chronic (4 μ M) gentamicin treatment conditions, the cytochrome c oxidase, DHE assays, and mitochondrial membrane potential assays were performed at 72 hours after optimizing the timing for these assessments. For assessment of STAT1 Ser⁷²⁷ and STAT1 Tyr⁷⁰¹ phosphorylation in the chronic (4 μ M gentamicin) treatment condition, explants were evaluated at 72 hours, with earlier time points selectively sampled if no gentamicin-induced change was noted at 72 hours. For assessment of STAT1 Ser⁷²⁷ and STAT1 Tyr⁷⁰¹ phosphorylation in the acute (50 μ M gentamicin) treatment condition, explants were initially

evaluated at 24 hours, with earlier time points sampled selectively since 50 μ M gentamicin induces diffuse hair cell death beginning after 24 hours.

Hair Cell Counts

At the experimental endpoints, explants were fixed with 4% paraformaldehyde overnight at 4°C and permeabilized with 3% Triton X-100 for 30 minutes in phosphate-buffered saline (PBS) at room temperature (22-24 °C). Explants were then washed with PBS three times, incubated with rhodamine-phalloidin (1:100) at room temperature for 30 minutes, and again washed three times with PBS. The explants were then mounted on a slide with FlouoroGel and examined under Leitz Orthoplan upright light microscope under 50x oil immersion lens. The right eye objective had a 0.19-mm calibrated scale superimposed on the visual field for reference. Specimens were oriented to show a single row of inner hair cells and all three rows of outer hair cells in the visual scale. Each 0.19 mm frame was evaluated for the presence or absence of inner and outer hair cells, starting from apex and moving down the base of the organ of Corti. The proportion of hair cells lost in each 0.19 mm frame was recorded in a computer program (KHRI cytochrome, version v.3.0.6, Kresge Hearing Research Institute, University of Michigan, Ann Arbor, MI, USA), allowing comparison with previously established normative data. The percentage of missing hair cells was plotted as a function of distance from the apical turn.

Immunofluorescence Assessment of Organ of Corti Explants

At the end of the desired time points, explants were fixed with 4% paraformaldehyde overnight at 4°C and permeabilized with 3% Triton X-100 for 30 min in phosphate-buffered saline (PBS) at room temperature (22-24°C). Explants were then washed with PBS three times, and incubated with STAT1 antibodies to the Tyr⁷⁰¹ or Ser⁷²⁷ phosphorylated residues, or to a common STAT1 target for measurement of total STAT1 prevalence (p-Stat1 Tyr⁷⁰¹, p-Stat1 Ser⁷²⁷, Stat1 p84/p91; Santa Cruz Biotechnology). Individual residue and total STAT1 phosphorylation was evaluated under “acute” high dose (50 μ M, 4-24 h) and “chronic” low dose (4 μ M, 16-72 h) gentamicin conditions using immunolabeling under confocal microscopy.

Dihydroethidium Assay for Superoxide Production/Suppression

Gentamicin-induced superoxide production (and potential attenuation by EGCG) was examined in explants treated with 50 μ M EGCG and/or 4 μ M gentamicin along with untreated controls. At the conclusion of the experiment, explants were stained with dihydroethidium (DHE; Sigma-Aldrich), and incubated for one hour. They were next fixed for 1 hour in 4% paraformaldehyde at room temperature, stained with 2 μ g/ml Hoechst stain (for nuclei) for one hour, mounted on slides, and examined immediately under confocal microscopy.

Evaluation of Mitochondrial Membrane Potential (after Gentamicin Administration)

Organ of Corti explants were harvested and exposed to 4 μ M gentamicin as detailed above. At 72 hours post-gentamicin, the cultures were exposed to 5,506,60-tetraethylbenzimidazolyl-carbocyanine iodide (DePsipher, Trevigen) for 30 min at 37°C.

When the mitochondrial transition pore is open, the potential-sensitive dye fluoresces Green (510/527 nm, corresponding to monomeric form). When inside an intact mitochondrion, the dye fluoresces Red (585/590 nm, corresponding to aggregated form). Imaging was performed immediately after 30 min dye-loading time to detect the ratio of aggregate/monomer for outer hair cells in each explant. Images confirmed disruption of mitochondrial membrane potential after gentamicin administration, which was preserved in untreated specimens.

Quantification of STAT1 Immunostaining

Automated thresholding and quantification of hair cell immunofluorescence was performed with ImageJ Java-based image processing (National Institute of Health, USA). Outer hair cells were registered using the analyzer function of ImageJ, and pixel value statistics of user-defined selections were measured with normalized intensity-threshold values. Data recorded for specimens by treatment condition allowed calculation of means with standard deviation and data were subjected to statistical analysis as below.

Statistical Analysis

Quantitative data were evaluated by Student's t-test and one-way Analyses of Variance (ANOVA) with Student-Newman-Keuls test for significance ($p < 0.05$) using SPSS Biostatistics software. For the relative fluorescence intensity, the protocol dictated two-tailed one-sample t-test using GraphPad Software (GraphPad Software Inc., San Diego, CA). For hair cell death, comparisons included cytochrome c data after treatment conditions.

Results

EGCG Reduces Gentamicin-Induced Outer Hair Cell Loss

The 4 μM gentamicin dose administered for 72 hours resulted in a $55 \pm 5\%$ outer hair cell loss, while administration of 50 μM EGCG beginning 1 hour before gentamicin therapy reduced outer hair cell loss to $10 \pm 7\%$ ($p < 0.001$). Cytochrome c data at 72 hours illustrates the outer hair cell loss that occurred with gentamicin and the relative sparing of outer hair cells when pre-treatment was performed with 50 μM EGCG. Compared to explants treated with gentamicin alone (**Figure 1, A**), explants pre-treated with 50 μM EGCG (**Figure 1, B**) demonstrate a rightward shift in curve profile, corresponding to a significant decrease in outer hair cell loss in the subset of gentamicin-treated explants that received 50 μM EGCG (**Figure 1, C**). Light microscopy images of hair cell morphology illustrate findings under these treatment conditions (**Supplemental Figure 1**). To optimize dosing with EGCG, a toxicity screen was completed at concentrations from 1 to 200 μM . Marked hair cell loss was noted at concentrations greater than 100 μM (data not shown).

Evaluation of STAT1 Prevalence and Phosphorylation after Gentamicin Challenge

STAT1 Tyr⁷⁰¹ and STAT1 Ser⁷²⁷ phosphorylation were examined under both high-dose gentamicin conditions (50 μM , 4-24 hours) and low-dose gentamicin conditions (4 μM , 16-72 hours) using separate antibody probes for each residue. In explants treated with 4 μM gentamicin, STAT1 Ser⁷²⁷ phosphorylation was significantly increased at 72 hours

compared to untreated controls. This increase in Ser⁷²⁷ phosphorylation at 72 hours was attenuated when the 4 μ M gentamicin therapy was preceded by treatment with 50 μ M EGCG (**Figure 2**). There was no significant increase in STAT1 Ser⁷²⁷ phosphorylation from the 4 μ M gentamicin treatment at earlier time points studied (**Supplemental Figure 2**). With the high-dose 50 μ M gentamicin regimen, there was no significant increase in STAT1 Ser⁷²⁷ signal at 4 hours from initiation of treatment, but a strongly increased signal emerged at 16 hours (**Supplemental Figures 3 and 4**). The pattern was different when probing for phosphorylation of STAT1 at Tyr⁷⁰¹. With the high-dose 50 μ M gentamicin regimen, there was a significant early increase in STAT1 Tyr⁷⁰¹ signal at 4 hours from initiation of treatment (**Supplemental Figure 5**). This STAT1 Tyr⁷⁰¹ signal diminished by 16 hours, however (**Supplemental Figure 6**), consistent with the distinct biological effects of STAT1 Tyr⁷⁰¹ and STAT1 Ser⁷²⁷ function.⁶ In explants treated with 4 μ M gentamicin, STAT1 Tyr⁷⁰¹ phosphorylation was not increased at any of the time points from 16 to 72 hours (**Supplemental Figure 7** and data not shown). Confocal images at 72 hours demonstrate minimal phosphorylated STAT1 Ser⁷²⁷ signal present in untreated control explants (**Figure 3A, B, and C**), similar to explants pretreated with EGCG alone (**Figure 3D**). Increased STAT1 Ser⁷²⁷ signal was present after 72 hours of 4 μ M gentamicin treatment (**Figure 3E**), and diffuse STAT1 Ser⁷²⁷ signal was present after 16 hours of 50 μ M gentamicin (**Figure 3F**). STAT1 Tyr⁷⁰¹ phosphorylation was not significantly elevated in controls or with 4 μ M gentamicin treatment (**Supplemental Figure 8 A-D, 72 hours**), whereas STAT1 Tyr⁷⁰¹ phosphorylation was significantly elevated after 4 hours exposure to 50 μ M gentamicin (**Supplemental Figure 8E**). After 24 hours exposure to 50 μ M gentamicin, STAT1 Tyr⁷⁰¹ signal was no longer increased, and severe injury to both hair cells and supporting cells was apparent (**Supplemental Figure 8F**).

All-trans Retinoic Acid Potentiation of Gentamicin-Induced Outer Hair Cell Loss

All-trans Retinoic acid (atRA) was administered at 0 μ M (control), 10 μ M or 100 μ M in combination with 4 μ M gentamicin. When gentamicin (4 μ M) was administered without atRA, $27 \pm 2\%$ outer hair cell loss was observed. With addition of 10 μ M atRA to 4 μ M gentamicin, outer hair cell loss increased to $44 \pm 8\%$. This outer hair cell loss further increased at 100 μ M atRA to $54 \pm 5\%$ (**Figure 4**). An increase in hair cell death was thus observed in combinations of gentamicin with increasing retinoic acid ($p < 0.001$), whereas retinoic acid alone up to 100 μ M did not demonstrate toxicity (data not shown). The morphology noted with the retinoic-acid/gentamicin combination was similar to that typically observed with high doses of gentamicin. Findings included increased hair cell death, altered hair cell shape, and disruption of stereocilia. Supernumerary hair cells were also present in the apex along with disorganization of stereocilia (morphology data not shown).

Effects on Superoxide production and Mitochondrial Membrane Potential

The effect of gentamicin administration on mitochondrial membrane potential and superoxide was evaluated at 72 hours. Mitochondrial membrane potential was preserved in untreated explants (**Figure 5C**), as shown by homogenous red label corresponding to aggregated form of voltage sensitive reagent. Dissipation of the mitochondrial membrane

potential with accompanying scattered hair cell loss was evidenced by predominant green labeling (monomeric form of reagent) in outer hair cells (**Figure 5D**). On DHE assay of superoxide production, 4 μ M gentamicin was found to induce a mosaic-type pattern of DHE staining, consistent with the variable time course with which adjacent hair cells may develop reactive oxygen species and/or proceed to apoptosis (**Figure 6B, F**). Pre-treatment of specimens with EGCG (50 μ M) attenuated this gentamicin-induced superoxide formation (**Figure 6D, 6H**), similar to untreated control explants (**Figures 6A, E**), and 50 μ M EGCG alone (**Figure 6C, G**).

Discussion

The sensory outer hair cells of the inner ear are the primary target of aminoglycoside ototoxicity, and there is no FDA-approved treatment to prevent such hearing loss. Experience with antioxidants has been encouraging,^{7,8} and we therefore investigated whether EGCG, which combines antioxidant effect with STAT1 inhibition, could confer protection. STAT1 inhibition has been suggested as a potential molecular target for protective therapy by virtue of its role in activating inflammatory and apoptotic pathways.⁹ The organotypic explant preparation, which is a powerful tool for testing putative otoprotectants and probing mechanisms, revealed evidence antioxidant activity as well as modulation of STAT1 activation.

While both cisplatin and aminoglycosides induce injury through ROS formation, the origin of ROS may differ. In cisplatin ototoxicity, NOX3 and xanthine oxidase have been implicated in the generation of superoxide. In contrast, ROS induced through aminoglycoside injury may come from different species and reactions entirely, including enzymatic and non-enzymatic causes. Mechanisms of aminoglycoside injury have been linked to Rho-GTPases and activation of the NADPH oxidase complex,¹⁰ mitochondrial dysfunction causing excessive ROS formation, or through gentamicin's role as an iron chelator to reduce molecular oxygen to superoxide radicals.¹¹ The diverse mechanisms through which a redox imbalance can be induced implicate multiple cellular pathways. .

EGCG has been studied as a protectant in several translational models of ischemia/reperfusion injury.¹²⁻¹⁴ EGCG has also been shown to suppress interferon gamma-induced phosphorylation of STAT1 at its Ser⁷²⁷ and Tyr⁷⁰¹ residues in cancer cells and to suppress cisplatin-induced phosphorylation selectively Ser⁷²⁷ in response to cisplatin in the cochlea.⁴ In aspiration-induced lung tissue injury, pre-treatment with EGCG reduced levels of cytokines (TNF α and IL-1), of activated STAT1 protein, and of phosphorylated protein levels of JAK1 and JAK2,¹⁵ which may be attributed to blockade of INF- γ induced STAT1 activation.¹⁶ Other studies emphasize EGCG's antioxidant properties by demonstrating cardioprotection against doxorubicin, an anthracycline aminoglycoside, by attenuating ROS production and increasing expression of endogenous antioxidant enzymes.^{17,18} In the present study, co-administration of gentamicin with atRA potentiated hair cell loss and death.

EGCG conferred significant protection from aminoglycoside-induced hair cell death in the explant model, associated with ROS suppression as well as decreased STAT1 Ser⁷²⁷

phosphorylation with the 4 μ M dose. Protection was associated with antioxidant properties, reflected in decreased superoxide on imaging of DHE. While STAT1 is up-regulated in cisplatin-induced hearing loss,¹⁹ STAT1 phosphorylation was a late and relatively subtle event in context of low dose gentamicin-induced injury. Elevation of STAT1 phosphorylation was observed at both residues at (less physiologic) high dose condition. Prior work found that STAT1 activation is necessary for p53-mediated killing by cisplatin in utricular hair cells based on work in STAT1-deficient mice, which were resistant to cisplatin toxicity.¹⁹ A recent study in the cisplatin ototoxicity literature found that the Adenosine A1 receptor agonist R-phenylisopropyladenosine (R-PIA) decreased cisplatin-induced phosphorylation of STAT1 Ser(727), but not STAT1 Tyr(701). R-PIA also reduced the expression of several STAT1 target genes, such as cyclooxygenase-2 (COX-2), TNF-alpha, and inducible nitric oxide synthase (iNOS) and was associated with decreased inflammation and apoptosis in the rat cochlea with preserved hearing.²⁰

IFN-gamma signaling induces phosphorylation at both Tyr⁷⁰¹ which is involved in dimerization, nuclear translocation and DNA binding as well as Ser⁷²⁷ which activates STAT1 transcriptional activity.⁶ In cisplatin injury models, where suppression of STAT1 through short interfering RNA reduced cisplatin induced ototoxicity in-vivo and attenuated reactive oxygen species.⁴ The preferential Ser⁷²⁷ phosphorylation noted after prolonged 4 μ M gentamicin therapy is seen in both cisplatin models as well as in UVB-induced injury,²¹ whereas interferon gamma activates both residues. These studies provide insight into the mechanisms through which EGCG confers protection. While EGCG is a STAT1 inhibitor, protection against gentamicin-induced hair cell death is also likely from improved redox balance in the face of cellular stress. Recent work comparing STAT1 wild type and STAT1^{-/-} mice found that while both cisplatin and gentamicin triggered inflammation and activated apoptotic events that were attenuated in knockouts, only gentamicin induced transient STAT3 tyrosine phosphorylation and Akt serine phosphorylation in STAT1^{-/-} explants.²² Such findings are of interest in light of recent work showing that EGCG acts through phosphoinositide 3-kinase (PI3K)/Akt signaling in cochlea neuronal stem cells to promote cell growth and neuron differentiation, which may be useful for the treatment of hearing loss.²³

The pre-treatment with 50 μ M EGCG reduced OHC death caused by 4 μ M gentamicin from 55% to 10%, and staining with DHE for superoxide detection illustrated a decrease in ROS in those explants pre-treated with EGCG. The use of atRA, which is a known STAT1 activator,^{5,24} did not induce cell death when given alone. Concurrent application with 4 μ M gentamicin potentiated gentamicin-induced hair cell death. Prior murine work has established that retinoic acid's ability to induce STAT1 is largely related to enriched levels of INF-receptor components or INF-regulated transcription factors.⁵ The increase in STAT1 Ser⁷²⁷ phosphorylation at 72 hours with 4 μ M gentamicin is a late event, which may reflect a less severe insult than occurs in cisplatin models of ototoxicity. HEI-OC1 cells, which are derived from the immortalized inner ear, can survive treatment with aminoglycosides despite induction of apoptotic pathways, whereas cisplatin leads to significant cell death.²⁵

Several limitations apply to this study. This study does not take into consideration the role of pharmacokinetics and metabolism that will affect outcomes if protectants are administered

orally for an in-vivo study. Achieving a therapeutic concentration in the inner ear is also limited by an intact blood-labyrinth barrier in-vivo, an obstacle that is not reflected in the explant model. The finding that high concentrations of EGCG can induce outer hair cell death loss is likely a reflection of removal of contributions from the blood labyrinthine barrier and first pass metabolism effects in the explant model. No clinical reports to our knowledge suggest ototoxic levels of the drug occur with oral supplementation. Retinoic acid, a STAT1 activator, potentiated gentamicin toxicity consistent with STAT1 activation and cell death gentamicin at 50 μ M doses. Future work may include examining protectants in an in-vivo model to determine if EGCG prevents against gentamicin induced hair cell loss.

Conclusions

EGCG attenuated gentamicin-induced injury to the sensory hair cells of the inner ear in a murine organotypic culture model. This protection appears to be mediated through EGCG's role as an antioxidant and inhibition of STAT1 activation, particularly the inhibition of phosphorylation at ser⁷²⁷, which was noted at 72 hours. The significance of the STAT1 activation by 50 μ M gentamicin is more difficult to interpret, given high dosage. EGCG attenuates gentamicin-induced superoxide production in cochlear explants, providing evidence for reactive oxygen species scavenging as a dual mechanism for protection from hair cell death. Gentamicin therapy is associated with dissipation of mitochondrial membrane potential. Prevention of gentamicin-induced ROS generation may be integral to preserving mitochondrial bioenergetics and a basis for hearing protection strategies aiming to promote inner ear sensory hair cell survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Attenuation of gentamicin toxicity by EGCG in explants at 72 hrs

(A) Cytochrome c histogram of explants treated with 4 μM Gentamicin alone, n=4, versus (B) those pretreated with 50 μM EGCG + 4 μM gentamicin, n=4. (C) Explants pretreated with EGCG (50 μM) demonstrated only $10 \pm 6.7\%$ OHC loss compared to $55 \pm 4.7\%$ OHC loss in gentamicin treated explants. (* denotes $p = 0.054$, ** denotes $p < 0.001$). See Supplemental Figure 1 for representative morphology.

Figure 2. Prevalence of phosphorylated STAT1 at Ser⁷²⁷ is increased after 72 hours of 4 μM gentamicin therapy, and this increase is attenuated by EGCG
 STAT1 Ser⁷²⁷ activation by 4 μM gentamicin at 72 hours in explants was significantly increased relative to all other groups including untreated controls, explants treated with 50 μM EGCG alone, and explants treated with 50 μM EGCG followed by 4 μM gentamicin exposure.

Figure 3. Representative morphology from basal turn demonstrating increased immunostaining for STAT1 Ser⁷²⁷ phosphorylation after gentamicin therapy

Sections show P2-P3 explants from cochlear base (red, Ser⁷²⁷ secondary antibody; green, rhodamine phalloidin; blue, DAPI nuclear stain). Controls include (A) untreated explant with secondary Ser⁷²⁷ antibody minus primary antibody and (B) 4 μ M gentamicin-treated explant also Ser⁷²⁷ minus primary antibody (B), confirming absence of nonspecific staining by Ser⁷²⁷ and showing expected outer hair cell injury in gentamicin group at 72 hours. This specimen is at junction of basal/mid turn, and note more injury is towards right (more basal) portion. Untreated control specimen (C) shows mild red fluorescent staining by Ser⁷²⁷, similar to 50 μ M EGCG alone (D). In gentamicin-treated specimens at 72 hours phosphorylation of STAT1 Ser⁷²⁷ residue is increased (E), although signal is stronger with high dose (50 μ M) gentamicin, shown here at 16 hours (F). At this 16 hour time point, morphology is still largely intact despite the high toxicity of 50 μ M gentamicin at later time points.

Figure 4. Retinoic acid, a STAT1 activator, potentiated gentamicin's toxicity in explant model Outer hair cell counts in explants treated with gentamicin (GM) alone (4 μ M), and GM + retinoic acid (atRA) (10 and 100 μ M) at 72 hours. Addition of either retinoic acid concentration caused a step-wise increase in OHC loss from $27 \pm 1.8\%$ in gentamicin alone treated condition to $44 \pm 8.0\%$ at 10 μ M, and furthermore to $54 \pm 4.9\%$ at 100 μ M. (* denotes p value of < 0.05 , ** denotes $p < 0.001$)

Figure 5. Dissipation of mitochondrial membrane potential after 72 hours of 4 μ M gentamicin in explant model

After treatment, explants were stained with DePsipher reagent and observed under confocal microscopy. Red stain is aggregated form of DePsipher reagent in intact mitochondria. This form reflects preservation of the mitochondrial membrane potential. Green is monomeric form, located outside of mitochondria. (A) control for aggregated state (red), (B) control monomeric states, (C) untreated explant demonstrates aggregated state indicating preserved mitochondrial membrane potential, (D) dissipation of mitochondrial membrane potential denoted by green, monomeric form with corresponding altered morphology.

Figure 6. Explants treated with 50 μM EGCG demonstrate a reduction in 4 μM gentamicin-induced superoxide production at 72 hours

Red stain is DHE (Dihydroethidium) for superoxide, and blue is Hoechst 33352 stain for cell nuclei.