Role of Hsp90/Akt pathway in the pathogenesis of gentamicin-induced hearing loss

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Abstract: Studies have suggested that gentamicin may induce hair cell apoptosis through the Hsp90/Akt signaling pathway. Nevertheless, the exact mechanisms remain unclear. The following study investigated Hsp90 expression in gentamicin-treated cochleae (in vitro and in vivo) and explored whether the Hsp90/Akt signaling pathway has a role in gentamicin ototoxicity. For in vitro experiments, organotypic cultures from post-natal organ of Corti, collected from post-natal day 2 or 3 (p2-3) CBA/J explants were treated with 0.2 mM gentamicin for 24 h; for the in vivo experiments, 6-week-old male CBA/J mice were injected with gentamicin (150 mg/kg) to induce hearing loss. P-Akt and Hsp90/AKT proteins expression and the levels of Hsp90-Akt complex were examined using immunochemistry and western blot. Our data suggested that Hsp90 expression decreased in the hair ear cells after treatment. In addition, the pAkt and Hsp90/AKT levels significantly decreased in treated mice compared to the control group. To conclude, these results support the idea that the Hsp90/Akt signaling pathway may have an important role in the ototoxic effects of gentamicin.

Keywords: Hair cells, apoptosis, hearing loss, Hsp90, gentamicin

Introduction

Heat shock protein 90 (Hsp90) is a cytoplasmic molecular chaperone which regulates protein folding and maintenance, as well as the degradation of misfolded proteins. Exposure of cells to environmental stress, such as heat, chemical, UV radiation pressure or mechanical stress, results in the expression of Hsps (Heat shock proteins) which regulates cell apoptosis. Moreover, Hsp90 inhibitors have been developed for cancer treatment by suppressing Hsp90 “client” proteins, such as AKT and phospho-AKT (pAKT) [1].

Hsp90 is essential for the proper functioning of Akt because it forms a chaperone-substrate protein complex; a reduction in Hsp90-Akt binding results in Akt inactivation [2]. Akt, also known as protein kinase B, regulates the phosphorylation of numerous downstream proteins and affects cell growth, cell survival, and cell differentiation. It is considered a pivotal anti-apoptotic factor in many different cell death paradigms [3, 4].

Considerable evidence has demonstrated that a decrease in protein kinase B (Akt) signaling pathways is associated with hearing loss and hair cell death in gentamicin-induced ototoxicity. For example, aminoglycoside treatment has shown to downregulate the phosphorylation of Akt on serine 473 (p-Akt S473) in sensory hair cells thus inducing permanent hearing loss and sensory hair cell loss [5]. Moreover, Heinrich et al. have found that gentamicin alters Akt-expression and its activated form (p-Akt) in the guinea pig cochlea [6]. Nevertheless, the exact mechanisms of gentamicin induced hearing and its activation of AKT have not been completely defined. During gentamicin-induced acute tubular injury, HSP90 is induced in the lysosomes and the nucleoli of damaged cells which then regulates the disposition of degenerated proteins and new protein synthesis which protects target cells from gentamicin nephrotoxicity [7].
Few studies have suggested that gentamicin induces hair cells apoptosis through regulation of Hsp90/Akt activation [8, 9]. We hypothesized that gentamicin would adversely affect the Hsp90/Akt signaling pathway and induce hair cell apoptosis.

**Materials and methods**

**Animals and gentamicin administration**

Experiments were conducted on male 6-week-old CBA/J mice with thresholds of auditory brainstem responses (ABRs) < 30 dB in the sound pressure level (SPL) and signs of middle ear infection on otoscopic examination. All the animals were housed in an environment with temperature of 22 ± 1°C, relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 hr with free access to water and diet. Gentamicin was administrated intraperitoneally at doses of 150 mg/kg (Invitrogen, USA) once a day for a duration of 10 days. The control group received sterile saline using the same injection route and treatment duration.

Treatment of explants

Explants were incubated for 2 days to recover from dissection stress before gentamicin treatment. The medium was then replaced with new media containing drugs and was incubated for 8-24 h. Final concentrations of 0.2 mM gentamicin were used.

**Auditory brainstem responses**

Before proceeding with euthanasia, all animals were assessed for their auditory function. Mice were anesthetized with isoflurane inhalation followed by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) for ABR tests. Body temperature was maintained at approximately 37°C by light heating. After deep anesthetization, acoustic stimuli were delivered monaurally to a Beyer earphone attached to a customized plastic speculum inserted into the ear canal. The positive needle electrode was inserted subcutaneously at the vertex while the negative and the ground electrodes were inserted in the mastoid area of the test ear and in the contralateral mastoid, respectively. ABR testing was performed in response to 8 kHz tone burst. The sound level began at 100 dB SPL and decreased in 5-Db steps until the response disappeared. Hearing threshold was determined by ABR waveforms and was defined as the lowest stimulus sound pressure at which wave I could be distinguished. All ABR measurements were conducted by the same experimenter. The ABR thresholds were assigned by an expert who was blinded to the treatment conditions.

**Tissue collection**

The animals were decapitated immediately after the final ABR measurement. The temporal bones were quickly removed and opened to expose the otic capsule. For immunohistochemical staining, the isolated cochlea was fixed in 4% paraformaldehyde overnight and then decalcified in 4% EDTA at room temperature with gentle agitation for 48-72 hours; the solution was not changed. For immunohistochemistry of cochlear paraffin sections, the specimens were dehydrated using graded concentrations of alcohol, embedded in paraffin blocks, and then sectioned into 4-μm-thick slices. For immunohistochemistry of cochlear surface preparations, each cochlea for immu-
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Immunohistochemistry of surface preparations was dissected under a microscope by removing the softened otic capsule, striavascularis, Reissner’s membrane, and tectorial membrane. The remaining tissue, including the modiolus and cochlear sensory epithelium was kept for later experiments of Immunohistochemistry. For western blot analysis, cochleae were collected and snap-frozen in liquid nitrogen, and then stored at -80°C.

**Immunohistochemistry for cochlear paraffin sections**

After deparaffinization and rehydration, sections were microwaved in 10 mM citric acid (pH 6.8) for antigen retrieval and incubated in 0.3% H2O2 for 10 min to block endogenous peroxidase activity. Samples were then incubated with diluted normal goat serum for 20 min to suppress nonspecific binding of immunoglobulin. Primary antibody was incubated overnight in a humid chamber at 4°C, followed by biotinylated secondary antibody (Vector, CA USA) for 30 minutes and ABC reagent (Vector, CA USA) for 30 minutes. Immunocomplexes of horseradish peroxidase were visualized by DAB (Dako, Glostrup, Denmark) reaction, and sections were counterstained with hematoxylin before mounting.

**Immunohistochemistry for cochlear surface preparations**

The whole mount preparations were permeabilized in 3% Triton X-100 solution for 30 minutes at room temperature. The specimens were washed three times with PBS and blocked with 10% goat serum for 30 minutes at room temperature. For immunolabeling of hair cell proteins, the tissues were incubated at 4°C for 24 hours with the following primary antibodies: monoclonal rabbit anti-HSP90 (1:50, Abcam #ab109248) and polyclonal rabbit anti-Phospho-Akt (Ser473) Antibody (1:50, Cell signaling #9271). After washing three times, the tissues were incubated with the Alexa Fluor 350- or 594-conjugated secondary antibody at a concentration of 1:200 at 4°C overnight in darkness. After washing, specimens were incubated with Alexa Fluor 488 phalloidin at a concentration of 1:100 for one hour in darkness. For the colocalization of AKT and HSP90, the specimens were first incubated with primary monoclonal mouse anti-HSP90, at 1:50 and the secondary antibody and then with primary polyclonal rabbit anti-AKT at 1:50 (Cell signaling #2920) and the secondary antibody. Following washing steps, the tissues were incubated with Alexa Fluor 594-conjugated secondary antibody at 1:200 overnight in darkness. For all immunolabeling samples, after the final wash with PBS, the tissue was dissected in PBS by removing the modiolus. The epithelia were divided into three segments (apex, middle, and base). Specimens were mounted on slides with Fluoro-gel with Tris buffer (Electron Microscopy Sciences #17985-10). Control incubations were routinely processed without primary antibody treatments. Immunolabeled images were taken using a laser confocal microscope (Zeiss LSM 510).

**Immunoprecipitation to assess for HSP90/AKT complexes**

Ten μg of well-vortexed Akt Mouse mAb (Sepharose Bead Conjugate, Cell signaling #3653) were mixed with 200 μg of total cochlear protein at 1 mg/ml in 1× Cell Lysis Buffer (10×). After incubation at 4°C on a rotating device overnight, the pellets were collected by centrifugation at 1,000× g at 4°C for 1 min. Consequently, the pellets were washed three times with lysis buffer and then re-suspended in 20 μL of electrophoresis buffer (including 0.5 M Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue and 10% β-mercaptoethanol). Western blotting was performed with an anti-HSP90 antibody. Six cochleae from 3 mice were pooled for each sample and total of at least 3 samples were used for each group.

**Western blot**

Total proteins from the cochlear basilar membrane were extracted using a total protein extraction kit, according to the manufacturer’s instructions. Protein concentrations were measured using a bicinchoninic acid protein assay kit. Proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Equal amounts of protein (20 μg) were loaded in each lane of the SDS gel. After transfer to nitrocellulose membrane, bands of HSP90 were detected using the primary antibody against HSP90 (Cell Signaling #4877) at a concentration of 1:1000. The amount of loading in each lane was verified using a mouse monoclonal antibody (ProMab).
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Figure 1. Gentamicin induces hair cell loss in the organ of Corti. A. Surface preparations of the entire length of the explant were evaluated quantitatively for hair cell loss as described in the “Materials and Methods” section. The percentage of missing OHCs increases with the period of incubation. At 16 h post-treatment, significant cell damage was observed in the treatment group compared to control group (\*P < 0.05, \**P < 0.01, \***P < 0.001; n = 3 at each time point). Data presented as average ± SD. B. Base-to-apex gradient of hair cell loss. Surface preparations of the basal segment show the outlines of IHCs and OHCs stained for F-actin with rhodamine phalloidin. Control incubations in the absence of gentamicin maintained a normal appearance at 24 h post-treatment. In the basal segment of the organ of Corti, OHCs loss became visible 16 h after incubation with gentamicin (hair cell loss are marked by arrowhead) and the damage increased over time. OHCs at the apex (GM 24 h Apex) remained intact.

Figure 2. Gentamicin downregulates HSP90 in Corti explants (basal turn). Outlines of IHCs and OHCs stained for F-actin with phalloidin-488 (Green). Compared to the control group, significantly lower HSP90 expression (red) was detected in OHCs 8 h post-treatment (one sample t test, t (3) = 30.317, P < 0.001). n = 4 at each time point.

Statistical analysis

Data were analyzed using SYSTAT and GraphPad software for Windows. Statistical methods used included one-way Analysis of Variance (ANOVA) with Tukey’s multiple comparisons, repeated-measures ANOVA with post-hoc testing, unpaired t-tests, and one-sample t-tests. All tests were two-tailed and p-value < 0.05 was considered significant.

Results

Gentamicin induces hair cell loss in vitro

After 8 h treatment with 0.2 mM gentamicin, no inner hair cell (IHCs) nor outer hair cell (OHCs) loss were observed; minimal loss in the basal segment were observed after 16 h of treatment. Significant hair cell death was detected after 20 h of treatment (Figure 1A). On the contrary, no cell damage was observed in the control group 24 h post-treatment (Figure 1B).
addition, gentamicin treatment (0.2 μM for 24 hours) damaged OHCs in a graded fashion; maximal damage was observed in the base of cochlea, while no OHCs were missing in the apex turn.

**Gentamicin reduces HSP90 expression in OHCs in vitro**

Although no hair loss was observed after incubation with 0.2 mM gentamicin for 8 h, the expression level of HSP90 decreased significantly in OHCs (the ratio is 4:3); significantly reduced levels remained until 16 h post-treatment (Figure 2).

**Hearing thresholds of ABR and hair cells loss in vivo**

ABR threshold data at 8.0 kHz were recorded for all the pre-recordings of the eight tested groups. Threshold levels significantly increased 10 days post-gentamicin injection (10.1 dB SPL, P < 0.05, n = 5). The cochlear basilar membrane revealed a small amount of IHCs and OHCs that were missing; however, no statistically significant differences were found (data not show).

**Down-regulation of pAKT expression upon Gentamicin ototoxicity in vivo**

Next, we examined the levels of Akt phosphorylated at S473 site on surface preparations of CBA/J mice after gentamicin exposure. Briefly, decreased levels of p-Akt (S473) were found in the treatment group compared to control group (ratio is 2.43:1, P < 0.01; n = 5) (Figure 3). In addition, western blot indicated that p-Akt (S473) levels of whole cochleae did not change after gentamicin treatment (P = 0.80, n = 5, data not shown).

**Down-regulation of HSP90 expression and HSP90- AKT complex upon gentamicin ototoxicity in vivo**

Localization and expression of HSP90 were investigated using anti-HSP90 immunostaining. HSP90 immunolabeling was detected throughout the cochlear basilar membrane, and immunoreactivity was found to be differentially distributed. Compared to the control group (Figure 4A), the expression of HSP90 significantly decreased in both IHC and OHC cells (Figure 4B).
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Only a single band was detected in homogenates of total cochlear tissue by western blot when probing with the HSP90 antibody. Quantitative analysis of the band densities showed a significant decrease between control and gentamicin exposure groups (Figure 5C). The ratio of the band densities of control was 0.63 (P < 0.05; n = 3). When probing individual cell populations by immunofluorescence, HSP90 also decreased in OHCs after gentamicin exposure (Figure 5A). Quantitative analysis of the HSP90-associated fluorescence in OHCs revealed a significant decrease. The ratio of relative fluorescence of control to gentamicin treatment samples was 1.84:1 (P < 0.01; n = 5). To sum up, these results indicated that HSP90 in OHCs was specifically associated with gentamicin exposure that induced permanent hearing loss.

AKT immunolabeling did not change after gentamicin exposure; nonetheless, decreased colocalization of HSP90 and AKT fluorophores were detected in OHCs (Figure 4A). Since we observed that HSP90, but not AKT, decreases in the inner ear after gentamicin exposure, we analyzed HSP90-AKT complexes by immunoprecipitating AKT from cochlear extracts and immunoblotting for HSP90. Gentamicin exposure significantly decreased the formation of HSP90- AKT complexes (Figure 5B). Quantitative analysis of the band densities of the HSP90- AKT complexes to total AKT showed a statistically significant decrease post-gentamicin exposure. The ratio of the band densities of controls was 0.66 (P < 0.05; n = 3).

Discussion

In the present study, the explant of the organ of Corti showed a base-to-apex progression of OHCs loss in response to gentamicin, consistent with previous studies [11]. Our data indicated that gentamicin induces evident hair cell damage both in vitro and in vivo.

In the course of eliminating the damaged cells by triggering apoptosis, initial cell responses resisted the drug action by invoking survival pathways. When damage overwhelms the repair capacity, cell death signaling and cell death signaling apoptosis occur. Therefore, the drugs can be initially present in hair cells without any measurable signs of pathology [12, 13]. This study demonstrated that HSP90 levels significantly decreased in the organ culture without obvious hair cell damage 8 hours after gentamicin treatment. We also found a significant decrease in both HSP90 and pAKT levels in OHCs, and no obvious hair cell damage after 10 days of gentamicin treatment in vivo. However, pAkt band densities were unchanged compared with controls in whole cochlear tissue homogenates by western blot. Also, ABR threshold levels were slightly and significantly increased, so the function (but not the structure) of hair cells may be affected and damaged at this time point.

Figure 5. Gentamicin exposure decreases the formation of HSP90/AKT by decreasing the expression of HSP90. A. Surface preparations illustrate the immunolabeling for the HSP90 (blue), the AKT (red) in OHCs with phalloidin staining (green), and the co-localization (pink) of HSP90- and AKT -associated immunofluorescence within OHCs (merged). All representative images were taken from the basal turn. Quantification of the HSP90 immunolabeling in OHCs after gentamicin exposure revealed a significant decrease in HSP90 (P < 0.01; t (4) = 6.2054), and no changes in AKT expression (P = 0.1215; t (4) = 1.9614). B. Gentamicin exposure decreases formation of HSP90/ AKT complexes. Complexes of HSP90 with AKT were detected by immunoprecipitation (IP) using an antibody against AKT, followed by immunoblotting (IB) with an antibody for HSP90. The membrane was then immunoblotted with an AKT antibody as a control for the amount of AKT in the immunoprecipitation assay. The band densities of complexes of HSP90/AKT were weaker after gentamicin treatment in comparison to the control group (P < 0.05; t (2) = 5.8371). C. Levels of HSP90 in the cochlear total protein extracts were detected by western blot and also showed a significant decrease (P < 0.05; t (2) = 5.7212). GAPDH was used as a loading control.

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To the best our knowledge, this is the first study that suggested the existence of a direct link between the gentamicin-induced hair cells apoptosis with the Hsp90/Akt pathway. To investigate potential mechanisms of pAkt decrease in gentamicin ototoxicity and/or the interaction between Hsp90 and Akt proteins, we determined whether gentamicin-treated altered Akt protein content and/or the Akt-Hsp90 complex level. In this study, we observed that Akt-Hsp90 complex level decreased with the decreased expression of HSP90, while there was no change in AKT expression.

Hsp90 contributes to the functional stabilization of Akt, activation of PI3K/Akt signaling pathway and cell survival. In addition, Hsp90 regulates Akt activity by inhibiting its phosphorylation and proteosomal degradation [14]. The Hsp90/Akt pathway is an important survival and anti-apoptotic pathway because the cleavage of Hsp90 in Akt-Hsp90 complex appears to be very important in the destabilization of the Akt-Hsp90 complex and in the triggering of apoptotic signals [15-18]. The formation of the Akt-Hsp90 complex stabilizes the Akt kinase activity and protects cells from undergoing apoptosis by preventing pAkt dephosphorylation [2].

Heat stress is effective at preventing hearing loss caused by exposure to excessive noise [8, 9], and heat shock has a significant protective effect against both aminoglycoside-and cisplatin-induced hair cell death [19]. These data indicate that heat shock may inhibit ototoxic drug-induced hair cell death. In this study, the expression levels of the Hsp90 protein were decreased in response to gentamicin-treatment, which then induced p-Akt degradation. Therefore, we can speculate that Hsp90 is involved in protection of gentamicin-induced hair cell death. Gentamicin can inhibit the expression of Hsp90, can contribute to the destabilization of the Akt-Hsp90 complex and increase p-Akt dephosphorylation, thus inducing hair cell apoptosis.

**Conclusion**

In this study, we found that the Hsp90/Akt signaling pathway has a role in the induction of gentamicin-induced apoptosis. Gentamicin induces ototoxicity by reducing the expression of Hsp90, and contributes to the destabilization of the Akt-Hsp90 complex, which in turn induces p-Akt dephosphorylation and hair cell apoptosis.

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**Disclosure of conflict of interest**

None.

**Abbreviations**

Hsp90, Heat shock protein 90; OHCs, Outer hair cells; IHCs, Inter hair cells.

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