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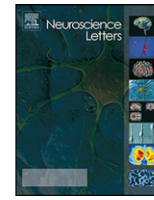

In vitro protection of auditory hair cells by salicylate from the gentamicin-induced but not neomycin-induced cell loss

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ABSTRACT

Salicylate has been shown to protect animals and people from the gentamicin-induced hearing loss. The objective of our study was to determine if salicylate is otoprotective *in vitro*. In this fashion, we wanted to validate the use of explant culture system for future studies on the ototoxicity prevention. In addition, we wanted to find out if salicylate protects from the ototoxicity of other aminoglycosides. As a model, we used the membranous cochlear tissues containing the organ of Corti, spiral limbus and spiral ganglion neurons dissected from the cochleas of p3–p5 Wistar pups. The explants were divided into apical, medial and basal parts and cultured in presence or absence of 100 μM gentamicin, 100 μM neomycin and 5 mM salicylate. Following the tissue fixation and staining with phalloidin–TRITC, the number of inner and outer hair cells (IHCs, OHCs) was scored under the fluorescent microscope. Presence of 5 mM salicylate in explants cultures exposed to 100 μM gentamicin significantly reduced the loss of IHCs and OHCs, as compared to explants exposed to gentamicin alone. In contrast, neomycin-induced auditory hair cell loss remained unaffected by the presence of salicylate. Our results corroborate earlier *in vivo* findings and validate the use of cochlear explants for future studies on ototoxicity and its prevention. Moreover, the inability of salicylate to prevent neomycin-induced ototoxicity implies possible differences between the mechanisms of auditory hair cell loss induced by gentamicin and neomycin.

1. Introduction

Increasing bacterial antibiotic resistance focuses once again the pharmacological and biomedical research on aminoglycoside antibiotics. Unfortunately, the side effects of aminoglycosides include *temporary* nephro- and *permanent* oto-toxicity [13]. Finding universal anti-ototoxic substance could considerably help avoiding detrimental side effects of aminoglycosides. This is of particular importance during treatment of multi-drug resistant *Mycobacterium tuberculosis* and treatment of opportunistic infections with *Pseudomonas aeruginosa* in patients with cystic fibrosis [10,11]. One of the anti-ototoxic strategies used by us and others was to induce expression of protective, anti-apoptotic protein HSP70 in the auditory hair cells [14,20,21]. Even simpler strategy seems to be the use of aspirin or its active compound—salicylate.

Aspirin and salicylate are themselves not free of ototoxic side effects and can cause *transient* tinnitus and *temporary* hearing loss [4,5]. Only forty years ago, aspirin-induced tinnitus was taken for a good sign of salicylate reaching therapeutic concentration in blood of the rheumatoid arthritis patients [9]. Work of Schacht and co-workers has demonstrated that administration of salicylate together with gentamicin and inhibit ototoxicity induced by this antibiotic [6,17]. The studies demonstrating otoprotective properties of salicylate were conducted either with use of animal model or as clinical study with gentamicin-exposed patients.

We were curious about the still unknown ability of salicylate to protect the auditory hair cells *in vitro* from gentamicin-induced ototoxicity. We reasoned that seeing a protective effect would not only validate sometimes disputed application of immature cochlear explant for the ototoxicity studies but would also facilitate future molecular analyses of the protective mechanisms.

The mechanism of ototoxicity induced by gentamicin and other aminogylcosides involves free radicals formation [13,16,18]. Interestingly, ototoxicity is exhibited by all aminoglycoside antibiotics but to a different degree [12,13]. Therefore, we asked if salicylate could induce general protective mechanism against other aminoglycosides or if the protection is limited to that against gentamicin.

2. Materials and methods

This study was performed in accordance with the German Prevention of Cruelty to Animals Act and was approved by the Berlin Senate Office for Health (T0234/00). Cochleae were dissected from

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postnatal Wistar pups (p3–p5) following the method of Sobkowicz et al. [19]. Seventy animals (140 cochleas) were used in total.

Dissected cochlear epithelium, which contained the organ of Corti and spiral limbus tissues with spiral ganglion neurons (the modiolus and stria vascularis with spiral ligament were removed) were washed carefully in buffered saline glucose (BSG) under sterile conditions. After that, the specimens were separated into apical, middle and basal parts. The explants were cultured in four-well microtiter plates (500 μl/well) in Dulbecco's modified Eagle's medium and Ham's F12 (1:1) (DMEM/F12, Gibco, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.6% glucose, 2 μl/ml insulin-transferrin-Na-Selenit-Mix (Roche Diagnostics GmbH, Mannheim, Germany), and 100 U/ml penicillin. All cultures were maintained at 37 °C in 5% CO_2 atmosphere. Culture medium was exchanged daily during the culture period, and each explant was cultured individually.

At the end of incubation, cultures were rinsed with phosphate buffered saline (PBS) and fixed for 30 min in 4% paraformaldehyde/0.1 M PBS (pH 7.4). After two rinses with PBS, explants were permeabilized with 0.2% Triton X-100 in PBS for 30 min. To visualize the hair cells, explants were incubated in a buffered solution containing TRITC-labeled phalloidin for 30 min at room temperature, as described by the group of Raphael [3]. After three rinses with PBS, explants were mounted with anti-fade solution for the epifluorescent microscopy.

Hair cells were counted under Leica DMIL fluorescence microscope, using 400 times magnification. Quantitation of inner hair cells (IHCs) and outer hair cells (OHCs) was performed in cochlear segments having length of 100 μm. In each cochlear segment analyzed, the hair cells were counted in three different fields.

For the statistical analyses, two-way ANOVA and Scheffé's post hoc test were used (Statistica 7.1, StatSoft). Differences with $p < 0.05$ were considered to be significant.

3. Results

To assess possible toxic effect of salicylate on the auditory hair cells, we performed titration experiments ($n = 10$ explants for each concentration). Three concentrations of salicylate (1, 2.5, and 5 mM) had no visible negative effect on the explant tissues, as per scoring the intact IHCs and OHCs and comparing the results to the cultures without salicylate. However, salicylate at the concentration of 10 mM induced significant IHC loss (Fig. 1A) but no OHC loss (Fig. 1B). In detail, 21, 14 and 19% of IHCs were lost in the apical, middle and basal part of the cochlea, respectively, as compared to the controls. Therefore, in all further experiments, 5 mM salicylate was used.

The epifluorescence micrographs in Fig. 2 show representative examples of phalloidin-stained explants: controls ($n = 20$) and explants exposed to 100 μM gentamicin alone ($n = 20$) or exposed to 100 μM gentamicin and 5 mM salicylate for 48 h. The controls (Fig. 2A–C) contained one intact row of IHCs (mean IHC number per 100 μm 9.70 ± 0.10 SEM; 9.65 ± 0.13; and 9.65 ± 0.13) and three rows of OHCs (mean OHC number per 100 μm 33.6 ± 0.35 SEM; 33.15 ± 0.30; and 32.85 ± 0.30) in the apical, middle and basal cochlear parts, respectively. Exposure to gentamicin induced significant loss of IHCs (mean IHC number per 100 μm was 5.85 ± 0.26 SEM; 6.0 ± 0.24; and 4.95 ± 0.26) and of OHCs (mean OHC number per 100 μm 22.0 ± 0.77 SEM; 16.10 ± 0.57; and 12.25 ± 0.57) in the apical, middle and basal cochlear parts, respectively (Fig. 2D–F). The gentamicin-induced hair cell loss was significantly reduced by salicylate in the IHCs (mean IHC number per 100 μm 8.30 ± 0.29 SEM; 7.7 ± 0.34; and 7.2 ± 0.22) and in the OHCs (mean OHC number per 100 μm was 29.90 ± 0.51 SEM; 26.10 ± 1.24; and 22.25 ± 0.58) in the apical, middle and basal cochlear parts, respectively (Fig. 2G–I).

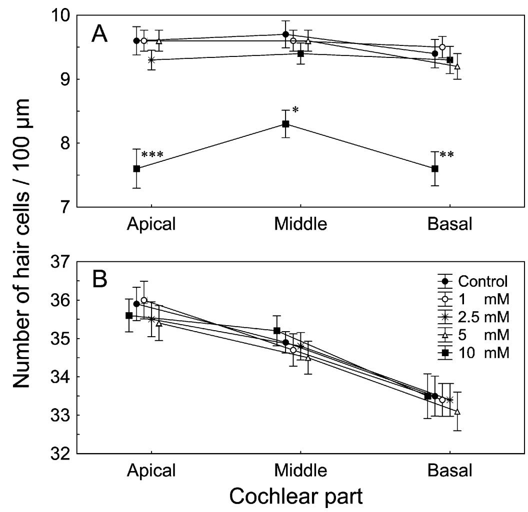

Fig. 1. Salicylate at concentrations of 1, 2.5 and 5 mM ($n = 10$ for each concentration) does not induce a loss of inner or outer hair cells (IHCs, OHCs) as compared to the controls. Salicylate at a concentration of 10 mM ($n = 10$) induces *in vitro* IHC loss (A), whereas the OHCs were not affected (B). The explants were cultured for 48 h in medium only or in presence of 5 mm salicylate. After that, the specimens were washed, fixed and stained with phalloidin–TRITC. Hair cells were counted under the epifluorescent microscope. Values are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ *vs*. controls.

The results of hair cell scoring after the exposure to gentamicin and treatment with salicylate are shown in Fig. 3. Following 48 h of *in vitro* exposure to 100 μM gentamicin, the loss of IHCs ranged from 38 to 49% (Fig. 3A) and the loss of OHCs ranged from 34.5 to 63% (Fig. 3B) as counted in the apical, middle and basal cochlear parts. Explants treated with 5 mM salicylate had reduced loss of IHCs ranging from 18 to 25% and of OHCs ranging from 23.5 to 30% (Fig. 3A and B).

To verify the protective ability of salicylate against other aminoglycosides, neomycin was used in the next set of experiments. Exposure to 100 μM neomycin for 48 h induced loss of 26, 52 and 58% of the IHCs (mean IHC number per 100 μm 7.10 ± 0.34 SEM; 4.60 ± 0.22; and 4.00 0.25, Fig. 4A) and 42, 42 and 84% of the OHCs (mean OHC number per 100 μm was 20.20 ± 0.96 SEM; 20.20 ± 1.06; and 5.60 ± 0.47, Fig. 4B) in the apical, middle and basal part of the explants, respectively. Simultaneous addition of 5 mM salicylate and 100 μM neomycin to the explant cultures had not significantly influenced the loss of the IHCs (mean IHC number per 100 μm 8.50 ± 0.37 SEM; 5.50 ± 0.37; and 3.80 0.24, Fig. 4A) or the OHCs (mean OHC number per 100 μm was 23.40 ± 1.23 SEM; 19.60 ± 0.63; and 6.20 ± 0.46, Fig. 4B) in the apical, middle and basal part of the explants, respectively.

4. Discussion

Aminoglycoside antibiotics are efficient anti-bacterial drugs, but their use is not free of risk of toxicity to kidney and permanent damage to the inner ear [12,13]. Our present study confirms the results obtained earlier by Schacht and his group with animal models or during clinical trials, demonstrating that the ototoxicity of gentamicin may be inhibited by simultaneous use of salicylate [15,17]. Although the design of *in vivo* and *in vitro* studies differed substantially, both types of studies showed that salicylate protected auditory hair cells [6,15,17].

The fact that the postnatal rats do not hear until two weeks after birth is often used as a major critique of results obtained with *in vitro* use of postnatal cochlear explants. Our results corroborate the *in vivo* effect; therefore, encourage further otoprotective studies with use of explants obtained from the immature cochlea.