Caspase-3 activation in the guinea pig cochlea exposed to salicylate

Hao Feng, Shi-Hua Yin, An-Zhou Tang, Hong-Wu Cai, Ping Chen, Song-Hua Tan, Li-Hong Xie

Department of Otorhinolaryngology-Head and Neck Surgery, First Affiliated Hospital of Guangxi Medical University, 22# Shuangyong Road, Nanning 530021, Guangxi, PR China

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ABSTRACT

In the current study, we explored whether chronic salicylate exposure could induce apoptosis in outer hair cells (OHCs) and spiral ganglion neurons (SGNs) of the cochlea. Guinea pig received sodium salicylate (400 mg/kg/d) or saline vehicle for 10 consecutive days. Programmed cell death (PCD) executioner was evaluated with immunohistochemistry detection of activated caspase-3. Apoptosis was examined with a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method. Repeated salicylate administration activated caspase-3 and caused apoptosis in OHCs and SGNs (p < 0.01 vs. saline control for both measures and in both cell types). Cell counting showed a significant loss in OHCs (p < 0.01 vs. saline control), but not in inner hair cells (IHCs). Transmission electron microscopy (TEM) revealed chromatin condensation and nucleus margination in salicylate-treated cochlea. Scanning electron microscopy (SEM) demonstrated stereociliary bundles breakdown and fusion at the apical of OHCs, villous matter was discovered to attach on the surface of SGNs. These findings suggest that long-term administration of high-dose salicylate can activate caspase-3 pathway to induce OHC and SGN apoptosis.

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Apoptosis is a normal physiological cell death which plays a critical role in maintaining tissue homeostasis. It has been proven that this particular pattern is of great importance to hearing system reorganization and maturity. Nishizaki et al. found apoptosis in the chick’s endolymphatic sac and vestibule at an early stage of embryonic development [15]. An apoptotic cell was also discovered in the middle ear, external auditory canal and acoustic neuroepithelium in prenatal mouse cochlea [14].

However, exposure to a variety of ototoxic factors has been shown to cause cochlea damage associated with excessive apoptosis. Ma et al. illustrated that apoptosis in the inner ear had been linked to sensorineural hearing loss (SNHL) in autoimmune inner ear disease (AIED) [12]. Watanabe et al. revealed that apoptosis played an important part in endolymphatic sac edema resulting from immune response in the guinea pig cochlea [19,20]. Yet they have not made further studies to clarify cochlear cell apoptosis control and regulation.

Caspase-3 has been suggested to be the primary executioner in most cellular apoptosis during both normal developmental cell death and the removal of damaged cells [1,17]. The results of gene knockout experiments in mice clearly show that the presence of a functional caspase-3 gene is required for the development of normal hearing [13,16]. In one histological analysis, the cochlear ducts obtained from 2-week-old caspase-3-deficient mice were found to be immature [16]. In these null mutant cochleae, there was hyperplasia of the supporting cells and progressive degeneration of sensory HCs that resulted in a severe impairment of hearing acuity. Analysis of caspase-3 null mutant mouse cochlea generated by another gene knockout experiment pointed out that, in addition to the progressive loss of both inner and outer hair cells, there was also a progressive postnatal loss of the auditory neurons from the spiral ganglion [13]. Based on these results, our goal is to investigate HCs and SGNs apoptosis and ultimate executioner caspase-3 activation in salicylate-treated cochlea in vivo, using guinea pig as an experimental model.

Twenty-two adult guinea pigs (body weight 300–350 g, male, no exposure to noise or ototoxic drug) provided by the Experimental Animal Center of Guangxi Medical University were employed for the present study. Twenty pigs were randomly divided into a saline control group and a salicylate group (Sinopharm., Shanghai, China; dissolved in 0.9% saline to be 0.1 g/l), 10 for each, then injected with a dose of 400 mg/kg/d for 10 consecutive days, respectively. After injection, 10 cochleae obtained from left ear in each group were collected for the next step; four cochleae were used for staining, four for EM, and the remaining two for HCs surface preparation. Those two remaining animals were treated with gentamicin (Pharm. Corp., Tianjin, China) under the same circumstances and used as activated caspase-3 positive control. Animals were maintained in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996) and the Policy of Animal Care and Use Committee of Guangxi Medical University.
Cochleae were perfused with 4% PFA (dissolved in 0.1 M PB buffer pH 7.4) through the round window and a hole made in the apical turn. They were then immersed in the same fixation for 48 h and decalcified using 10% EDTA (dissolved in 0.1 M PB buffer pH 7.4) for 3–4 weeks with daily changing.

Paraffin-embedded tissue sectioned at a thickness of 5 μm was incubated with primary antibody against rat caspase-3 protein (Boster, Wuhan, China) for 24 h after deparaffinating and hydration. HRP-conjugated anti-rabbit Ig-G (Maixin, Fuzhou, China) were used as secondary antibody to link primary antibody. Then the specimen was stained with DAB and counterstained with hematoxylin. Sections incubated with PBS instead of primary antibody were used as negative control, while specimen from gentamicin-treated cochlea served as positive.

Fig. 1. A non-specific small dot-staining pattern was seen throughout the whole cochlea in the saline control (A). Correspondingly, TUNEL-positive cell was absent (B). Sections from animals disposed with salicylate showed an intensive pigmentation of activated caspase-3 in the cytosol of HCs and SGNs (a), immunoreactivity in the nucleus was detected in OHCs and SGNs, but not in IHCs or other supporting cells (b). Positive staining cell was marked by arrow. Scale bars = 30 μm for SGNs, 50 μm for coil of cochlea and 20 μm for HCs, respectively.

Fig. 2. Negative and positive control. Scale bars = 30 μm for SGN and 20 μm for HC, respectively.
For TUNEL assay, sections were incubated with an apoptosis mixture (Zhongshan, Beijing, China) for 1 h, including 5 μl of TUNEL enzyme and 45 μl of label solution. 50 μl of POD-Converter was added after PBS washing. The nuclei were stained with DAB and counterstained with hematoxylin. Sections exposed to PBS instead of reaction mixture served as negative control, while the positive control was exposed to 20 U DNase I (10 U/ml, dissolved in 50 mM Tris–HCl buffer pH 7.4) for 1 h to induce DNA strand break prior to the previous labeling procedures.

Cochleae were perfused and immersed in 2.5% GA (dissolved in 0.1 M PB buffer pH 7.4) for 2 h at room temperature. They were then dissected under a stereomicroscopy (SZ61, Olympus, Tokyo, Japan) to expose the organ of Corti (OC) and modiolus. After dehydration in graded alcohols, the specimens were dried by the critical point method, mounted on stubs with double-coated adhesive tape and covered with a thin layer of gold palladium in an anion sputter coating unit. A SEM (JSM-T–300, Jeol, Tokyo, Japan) was used to examine the specimens. Procedures for TEM (Hitachi-H-500, accelerating voltage 75 kV, Hitachi, Tokyo, Japan) were the same as SEM in general, except that ultrathin sections at a thickness of 60 nm were stained with uranyl acetate and lead citrate after post-fixation.

Cochleae were quickly removed and perfused with a silver nitrate (dissolved in double distilled water to be 0.5%) and 2.5% GA solution. Staining cochleae were dissected from apex to base, mounted in sections in glycine and examined under a light microscopy. HCs without stereociliary were considered to be lost and were not included in the counts. HCs number was quantified per 1 mm length of the basilar membrane using an ocular grid system and a 20× objective lens.

Five sections at an interval of 20 μm from the same cochlea, 20 in total, were photographed and analyzed using a Leica System (DMIRQ550, Leica, Wiesbaden, Germany). Average optical density (AOD) was used to evaluate antigen expression. TUNEL staining to identify cell degeneration was assessed by apoptosis index (AI). All data were expressed as mean ± SD, and comparisons between two groups were performed using the unpaired Student’s t-test, p < 0.01 was considered to be statistically significant. The data were analyzed with SPSS 13.0 software (SPSS Science, Chicago, U.S.A.).

No specific staining was detected in the saline control (Fig. 1A), accompanied by the absence of TUNEL-positive cell (Fig. 1B). Caspase-3 immunoreactivity was identified in the cytosol of HCs and SGNs after salicylate injection (p < 0.01) (Fig. 1A). Accordingly, TUNEL-positive HCs and SGNs emerged (p < 0.01) (Fig. 1B). No cytosol or nuclear staining was seen in the negative control (Fig. 2A and C). On the contrary, positive control was full of staining cells (Fig. 2B and D). The semi-quantitative analysis of activated caspase-3 expression in HCs and SGNs was figured out in Fig. 3A. Apoptosis index (AI) in HCs and SGNs was figured out in Fig. 3B.

No morphological change occurred in the saline control (Fig. 4A–D), while OHCs and SGNs obtained from salicylate-disposed cochlea displayed apoptotic changes, characterized with chromatin condensation and nucleus margination (Fig. 4a and d), and vacuolization in the cytosol (Fig. 4d). Waviness along the lateral wall in the OHCs was also exhibited (Fig. 4d). In addition, villous matter was found to attach on the surface of SGNs (Fig. 4b). Interestingly, no significant damage was seen in the IHCs disposed with salicylate (Fig. 4c). Normal V-shaped stereociliary bundles showed staircase morphology by SEM (Fig. 4E). Salicylate initiated stereociliary bundles breakdown and fusion at the apical of OHCs, while the IHCs retained intact just with a little chaos in the bundles (Fig. 4e).

Normal V-shaped stereociliary at the apical of OHCs were evident by silver nitrate staining (Fig. 5A). Scattered loss in the OHCs was obvious in the salicylate-treated cochlea, especially in the third row (Fig. 5B), while the IHCs at the single row remained intact. The mean percentage losses of IHCs and OHCs in the two groups were statistically analyzed and shown in Fig. 5C and D.

It is well known that salicylate has an effect on the auditory system, especially for HCs and SGNs, via a wide range of pharmacological target sites, such as Ca2+, K+ and Na+ voltage-gated channels [5]. Further study indicated that salicylate broke neurotransmitter balance in the inner ear and confused nerve impulses as a result of excitatory neurotransmitter up-regulation and/or inhibitory neurotransmitter down-regulation [3–5], which could result in a specific protein abnormality. Caspase-3 activation is a key event that mediates apoptosis regardless of various causes, which has already been noted in the inner ear after treatment with lipopolysaccharide [18] and transtympanic injection of gentamicin [7]. Some experiments revealed that caspase inhibitors prevented cisplatin-induced apoptosis of auditory sensory cells [9]. In our study, salicylate aggravated caspase-3 activation, and therefore resulted in apoptosis in auditory hair cells and neurons. To be specific, TUNEL-positive staining in the OHCs and SGNs increased a lot accompanied by activated caspase-3 up-regulation. Interestingly, IHCs showed activated caspase-3 expression with no TUNEL-positive staining detected. Other research found that salicylate had a tendency of inducing SGNs to be degenerated in an organotypic culture assay; whereas HCs in the same culture appeared normal comparatively [22]. So we concluded that HCs was more tolerant than SGNs in the damage induced by salicylate, and IHCs was tolerant than OHCs.

Degeneration of outer hair cells and hemorrhages in perilymphatic spaces were reported in the early anatomical observations [11]. Covell indicated vacuolization of outer hair cells and distension of the stria vascularis [2]. Falbe-Hansen [6] and Gottlib [8] demonstrated alterations in cells of the spiral ganglion in succession. In the present experiment, results on morphological data revealed that submicroscopic changes obviously occurred in the
Fig. 4. EM observation for SGNs and HCs. No morphological change occurred in the saline control (A–D). The salicylate-intoxicated neurons demonstrated apoptotic changes, characterized with chromatin condensation and nucleus margination (white asterisk). The mitochondria in the cytosol appeared an image of vacuolization (white box) (a). Villous matter was discovered to attach on the surface of SGNs (b). Salicylate induced changes in OHCs lateral wall, featured with waviness (black arrow) (d and d'), while IHCs remained intact perfectly, with integrated tight junction (black arrow) and normal synapse at the basal pole (black box) (c). Montages of SEM image from cochlea with salicylate disposal revealed stereociliary bundles breakdown and fusion at the apical of OHCs (white box), accompanied by loss in the third row (white asterisk) (e), while IHCs remained intact just with a little chaos in the bundles (white box). Normal V-shaped stereociliary bundles showed staircase morphology in the saline control (E). Scale bars = 1 μm for A, b, d and d'; 500 nm for the box at the top left of A and a; 10 μm for B, E and e; 2.5 μm for C and c; 0.5 μm for D.
cochlea disposed with salicylate and characterized with chromatin condensation and nucleus margination. In particular, villous matter was discovered to attach on the cell body of neurons, and we summarized that it had an impact on action potential (AP) generation. Work by Lue and Brownell found salicylate induced OHCs spontaneous activity bursts and vacuolization located at the bottom of OHCs connected to interior olivocochlea bundle [10]. Combined with our observation, we believed that salicylate could do harm to acoustic-electric transducer focusing on morphological change. Base on alterations in the four regions of OHCs, stereocilia bundles breakdown at the apical surface, decrease of synapse ribbon and vesicle at bottom, recession of organelle in the cytoplasm, and waviness along the lateral wall.

Besides, HCs preparation obtained from salicylate-disposed cochlea showed a significant loss in the OHCs with a rate of no more than 20% in the apical turn, especially at the third row, while HCs at the single row escaped from loss. Woodford et al. injected five chinchillas with an intramuscular injection of salicylate at a dose of 400 mg/kg, and discovered a small loss of slightly more than 10% of outer hair cells in a restricted area of the upper cochlea. They concluded that this loss could be attributed to salicylate [21]. In summary, we supposed that slight injury on sensory structure was quite coincidence with the mildness and reversibility of the perceptive alterations induced by salicylate.

In conclusion, this study demonstrates that cochlea impairment induced by salicylate is attributed to caspase-3-dependent apoptotic pathway. In addition, salicylate could definitely induce submicroscopic change in HCs and SGNs. So we suppose that blocking apoptosis at the caspase level might rescue auditory hair cells and neurons.

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References


