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SALICYLATE-INDUCED DEGENERATION OF COCHLEA SPIRAL GANGLION NEURONS-APOPTOSIS SIGNALING

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Abstract—Aspirin, whose active ingredient is sodium salicylate, is the most widely used drug worldwide, but it is not recommended for children because it may cause Reye's syndrome. High doses of salicylate also induce temporary hearing loss and tinnitus; while these disorders are believed to disappear when treatment is discontinued some data suggest that prolonged treatment may be neurotoxic. To investigate its ototoxicity, immature, postnatal day 3 rat cochlear organotypic cultures were treated with salicylate. Salicylate did not damage the sensory hair cells, but instead damaged the spiral ganglion neurons (SGN) and their peripheral fibers in a dose-dependent manner. The cross-sectional area of SGN decreased from 205 μm^2 in controls to 143, 116, and 91 μm^2 in cultures treated with 1, 3, or 5 mM salicylate, respectively. Morphological changes and caspase upregulation were indicative of caspase-mediated apoptosis. A quantitative RT-PCR apoptosis array identified a subset of genes up- or down regulated by salicylate. Eight genes showed a biologically relevant change ($P<0.05$, ≥2 fold change) after 3 h treatment with salicylate; seven genes (Tp53, Birc3, Tnfrsf5, Casp7, Nfkb1, Fas, Lta, Tnfsf10) were upregulated and one gene (Pycard) was downregulated. After 6 h treatment, only one gene (Nol3) was upregulated and two genes were downregulated (Cideb and Lhx4) while after 12 h treatment, two genes (Il10, Gadd45a) were upregulated and 4 (Prok2, Card10, Ltbr, Dapk1) were downregulated. High doses of salicylate in a physiologically relevant range can induce caspase-mediated cell death in immature SGN; changes in the expression of apoptotic genes particularly among members of the tumor necrosis factor (TNF) family appear to play an important role in the degeneration. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: aspirin, tumor necrosis factor, organotypic culture, PCR-array, caspase, NF-κB.

Sodium salicylate (SS), the active component of aspirin, is one of the most widely used over-the-counter drugs for the treatment of pain, fever, inflammation, heart attacks, chest pain, and a host of other health conditions (Lewis et al., 1983; Levine et al., 2005; Cheng, 2007). Aspirin has been implicated Reye's syndrome, a childhood disorder characterized by hyperammonemia and encephalopathy, and which may involve mitochondrial permeability transition (Trost and Lemasters, 1996; Larsen, 1997). Long-term consumption of high levels of SS can induce stomach bleeding, heart burn, nausea, and vomiting (Levine et al., 1986). At high doses, SS can induce hearing loss and tinnitus, a phantom auditory sensation (Waltner, 1955; Vonweiss and Lever, 1964; Myers and Bernstein, 1965; Myers et al., 1965; Boettcher and Salvi, 1991). Although the ototoxic effects of salicylate have been well documented in humans and other mammals (Cazals, 2000; Yang et al., 2007), the biological mechanisms responsible for these disorders remain poorly understood. High doses of SS can impair neuronal function at multiple sites along the auditory pathway beginning with the outer hair cells (OHC) and spiral ganglion neurons (SGN) in the cochlea and progressing centrally to the auditory cortex (AC) (Chen and Jastreboff, 1995; Kakehata and Santos-Sacchi, 1996; Gao, 1999; Peng et al., 2003; Basta and Ernst, 2004; Wang et al., 2006; Basta et al., 2008). It is widely believed that the ototoxic effects of SS are temporary and completely reversible in adult animals (McFadden et al., 1984; Jung et al., 1993; Puel, 2007). This view is based largely on the fact that SS-induced hearing loss (audiometric threshold) and tinnitus seem to recover 24–48 h after cessation of treatment. However, recent *in vivo* studies from our lab suggest that chronic treatment with high levels of SS sufficient to induce tinnitus and hearing loss permanently reduces the neural output of the cochlea (Chen et al., 2010). Previous *in vitro* studies indicate that SS can damage SGN neurites although the mechanisms underlying this neurotoxic effect are unknown (Zheng et al., 1997). Degeneration of SGN neurites occurs with salicylate concentrations similar to those found in the cerebral spinal fluid of rats with behavioral evidence of tinnitus (Jastreboff et al., 1986). Given aspirin's widespread use (Green, 2001; Heard et al., 2008) and its putative contribution to the encephalopathy associated with childhood Reye's syndrome (Larsen, 1997; Lemberg et al., 2009), there is substantial interest in determining whether the high doses of SS that induce tinnitus and hearing loss also lead to SGN degeneration. As a starting point for addressing this question, we applied SS to immature, postnatal cochlear organotypic cultures and evaluated the morphological changes and changes in apoptosis gene expression at 3, 6, and 12 h post-treatment.

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Abbreviations: AC, auditory cortex; BME, basal medium Eagle; CAD, caspase-activated DNase; CAP, compound action potential; Ct, cycle threshold; DISC, death-inducing signaling complex; FADD, Fas-associated protein with the death domain; Fas-L, Fas ligand; IAP, inhibitor of apoptosis; ICAD, inhibitor of caspase-activated DNase; IHC, inner hair cell; OHC, outer hair cell; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SGN, spiral ganglion neurons; SS, sodium salicylate; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

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EXPERIMENTAL PROCEDURES

Cochlear cultures

Cochlear organotypic cultures were prepared from postnatal day 3 SASCO Sprague–Dawley rats as previously described (Ding et al., 2002; McFadden et al., 2003). Following decapitation, the cochlea was carefully removed and the organ of Corti and SGN were isolated and transferred onto rat tail collagen gel (type I rat-tail collagen (Collaborative Research, 3.76 mg/ml in 0.02 N acetic acid), 10×BME (basal medium Eagle) with 2% sodium carbonate in a 9:1:1 ratio). A 15-μL drop of the collagen gel was place onto the surface of a 35-mm culture dish and allowed to gel for approximately 30 min. Afterwards, 1.3 ml of culture medium (0.01 g/ml bovine serum albumin (BSA, Sigma A-4919), 1% Serum-Free Supplement (Sigma I-1884), 2.4% of 20% glucose (Sigma G-2020), 0.2% penicillin G (Sigma P-3414), 1% 200 mM glutamine (Sigma G-6392), 95.4% of 1×BME (Sigma B-1522)) was added to the dish. The cultures were subsequently transferred to an incubator (Forma Scientific 3029, 37 °C, 5% CO_2) for overnight incubation. On the following day, culture medium was replaced with new medium containing the desired compounds for the experimental treatments.

Salicylate treatment

At the start of the salicylate treatment, normal culture medium was removed and replaced with medium containing 0.01 g/ml bovine serum albumin (BSA, Sigma A-4919), 1% Serum-Free Supplement (Sigma I-1884), 2.4% of 20% glucose (Sigma G-2020), 0.2% penicillin G (Sigma P-3414), 1% 200 mM glutamine (Sigma G-6392), 95.4% of 1×BME (Sigma B-1522), and various concentrations of salicylate (Sigma 71943, control, 1, 3, 5 or 10 mM). Afterward, the cultures were placed in an incubator (Forma Scientific 3029, 37 °C, 5% CO_2) for a specified treatment period. At the end of the treatment, the cochlear cultures were removed from the dish and fixed with 4% paraformaldehyde for 1 h and the tissue and collagen gel base were removed from the culture dish for staining and analysis.

Staining

Cochlear cultures were rinsed in 0.1 M PBS and immersed overnight at 4 °C in a solution containing a monoclonal antibody against neuronal class III β-tubulin (Covance, MMS-435P). The antibody was diluted 1:100 in blocking solution (Triton X-100 (1%), goat serum (3%), and 0.1 M PBS (96%)). The specimens were rinsed with 0.1 M PBS three times and incubated for 1 h with a secondary antibody labeled with Cy3 (goat anti-mouse IgG, Jackson ImmunoResearch Code: 115-165-206) dissolved in 0.1 M PBS (1:300). The specimens were rinsed with 0.1 M PBS and stained with Alexa 488-labeled phalloidin (Invitrogen A12379, diluted by 1:200) for 30 min. Specimens were rinsed with 0.1 M PBS and mounted on glass slides in glycerin and coverslipped.

A Polycaspase Assay Kit (Green, Neuromics, KF17200) was used to detect caspase activity (caspase-1, −3, −4, −5, −6, −7, −8, and −9). For SGN culture dish, 1 ml of serum-free culture medium with or without 5 mM SS was added to the treatment and control cultures, respectively. Cultures were incubated for 3 h followed by addition of 34 μl of 30× FAM-FLICA (1:30) working solution and incubation for 1 h. Specimens were then transferred to tubes with 0.9 ml freshly prepared 1× wash buffer and washed two times. Tissues were fixed for 30 min in 10% fixative (included in the kit). Specimens were then stained with a monoclonal antibody against neuronal class III β-tubulin as described above.

Confocal microscopy

Specimens were examined with a confocal microscope (Zeiss LSM-510 meta, step size 0.5 μm per slice). For examination of SGN somas, 20–40 image planes were typically acquired; for hair cells and nerve fibers, 60–100 image planes were typically acquired. Unless specified, six samples or more were prepared for each experimental condition. Representative photomicrographs illustrating typical morphology were taken from the middle of each basilar membrane specimen. Images from multiple layers were projected onto a single plane using the Zeiss LSM Image Examiner (version: 4,0,0,91).

To quantify the shrinkage of SGN, the size of all SGN somata were calculated using the Zeiss LSM Image Examiner as follows. Multiple layers of the images were first merged onto a single layer. The number of layers merged was determined using two criteria: (1) the number of layers was maximized so that the largest cross sectional area of each SGN was included in analysis and (2) the overlap among different SGN was minimized. A polygon was drawn around the perimeter of the cell body of all distinguishable SGN and the Zeiss LSM Image Examiner (version: 4,0,0,91) automatically calculated the enclosed area. Data were evaluated for statistical significance with SigmaStat (version 3.5.0.54).

Cochleograms

Hair cell counts were obtained from cochlear cultures treated with 5 mM SS and from controls. Cochleograms were prepared as described previously (Ding et al., 2001). Briefly, specimens were fixed with 4% paraformaldehyde for 1 h, stained with Alexa 488-labeled phalloidin and examined under a fluorescence microscope (Zeiss Axioskop). The number of missing IHC and OHC were counted over 0.24 mm intervals along entire the length of the cochlea. Using lab norms (Ding et al., 2007), a cochleogram showing the percent hair cell loss as a function of percent distance from the apex was constructed for each sample. Results from controls and the 5 mM SS group were averaged (n=6/group) to obtain a mean cochleogram.

Quantitative RT-PCR

The SGN were carefully isolated from the organ of Corti and transferred into culture dishes containing culture medium (see above) with or without 5 mM SS. The numbers of cultures for each condition were 3 h control (10), SS (12); 6 h control (30), SS (32); 12 h control (44), SS (44). Cultures were placed in an incubator for 3, 6, or 12 h. Samples were harvested subsequently and total RNA (tRNA) was isolated and purified (RNeasy Lipid Tissue Mini Kit, QIAGEN 74804). Each sample of purified tRNA was diluted 1:100 in RNase-free water and examined on a spectrophotometer (Beckman Coulter DU640 Spectrophotometer; 260 nm/230 and 260 nm/280 nm) to test the purity and concentration (μg/ml) of RNA used for synthesis of first-strand complementary DNA (cDNA; RT^2 First Strand Kit, SABiosciences, C-03). The concentration of tRNA was used to ensure a consistent amount of tRNA (0.4 μg in this experiment) was used for cDNA production across all experimental conditions.

Expression of 84 apoptosis-related genes was evaluated in control and SS-treated (5 mM, 3, 6, or 12 h duration) SGN samples in a 96-well plate (including five housekeeping and seven control genes; Rat Apoptosis RT^2 Profiler™ PCR Array, SABiosciences, PARN-012A). Apoptosis gene arrays were processed according to the manufacturer's instructions. RT2RealTimeTM SYBR Green/fluorescein PCR Master Mix (included in the kit) was used to monitor the fluorescence signal during each cycle of the PCR reaction. The apoptosis arrays were evaluated on a MyiQ™ Single-Color Real-Time PCR Detection System (BIO-RAD, Model No. MyiQ™ Optical Module). Each PCR reaction started with an initial denaturation cycle at 95 °C for 10 min, followed by 40 cycles consisting of 15 s at 95 °C for denaturating and 1 min at 60 °C for annealing. Each experimental condition was repeated three times. The threshold for calculating cycle threshold (Ct) values was calculated using MyiQ software (version: 1.0.410)

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