Bcl-2 Genes Regulate Noise-Induced Hearing Loss

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Proteins of the Bcl-2 family have been implicated in control of apoptotic pathways modulating neuronal cell death, including noise-induced hearing loss. In this study, we assessed the expressions of anti- and proapoptotic Bcl-2 genes, represented by Bcl-xL and Bak following noise exposures, which yielded temporary threshold shift (TTS) or permanent threshold shift (PTS). Auditory brainstem responses (ABRs) were assessed at 4, 8, and 16 kHz before exposure and on days 1, 3, 7, and 10 following exposure to 100 dB SPL, 4 kHz OBN, 1 hr (TTS) or 120 dB SPL, 4 kHz OBN, 5 hr (PTS). On day 10, subjects were euthanized. ABR thresholds increased following both exposures, fully recovered following the TTS exposure, and showed a 22.6 dB (4 kHz), 42.5 dB (8 kHz), and 44.9 dB (16 kHz) mean shift on day 10 following the PTS exposure. PTS was accompanied by outer hair cell loss progressing epidurally and basally from the 4-kHz region. Additional animals were euthanized for immunohistochemical assessment. BcL-xL was robustly expressed in outer hair cells following TTS exposure, whereas Bak was expressed following PTS exposure. These results indicate an important role of the Bcl-2 family proteins in regulating sensory cell survival or death following intense noise. Bcl-xL plays an essential role in prevention of sensory cell death following TTS levels of noise, and PTS exposure provokes the expression of Bak and, with that, cell death.

Key words: inner ear; hearing; guinea pig; apoptosis; Bcl-xL; Bak

Apoptosis plays an important role in metabolic stress-induced neuronal cell death, including sensorineural cell death in the auditory system induced by intense noise exposure (Hu et al., 2002; Wang et al., 2003; Lang et al., 2006). Thus direct mechanical trauma induced by intense noise exposure may contribute to cochlear sensory cell death involving necrosis or apoptosis (Yang et al., 2004; Hu et al., 2006). Noise exposure also causes hearing loss and sensory cell death resulting from metabolically initiated changes, including formation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radical species (Ohlemiller et al., 2000; Van Campen et al., 2002; Coling et al., 2003; Ohinata et al., 2003; Yamashita et al., 2004a; Henderson et al., 2006). These free radicals may directly cause the destruction of cellular and subcellular elements and may also serve as signaling molecules to up-regulate genes responsible for cell survival and apoptotic cell death. Recent studies of morphological characteristics and biochemical events in sensorineural elements of the inner ear following noise exposure have indicated nuclear condensation, DNA fragmentation, cell shrinkage, and activation of endonucleases consistent with apoptotic processes underlying noise-induced hearing loss (NIHL) and driving hair cell and auditory nerve death (Nicotera et al., 2003; Yamashita et al., 2004b; Vicente-Torres and Schacht, 2006).

Many molecules have been identified as executing factors or key modulators in apoptosis. Among them, proteins of the Bcl-2 family principally control the mitochondrial-initiated apoptotic pathway (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000a). Bcl-2 family members act upstream of caspase activation and serve as checkpoints in the regulation of apoptosis (Green and Kroemer, 2004). The Bcl-2 family includes two groups: antiapoptotic members include Bcl-2 and Bcl-xL, and proapoptotic members include Bax and Bak. These proteins form homodimers, and the ratio of antiapoptotic to proapoptotic Bcl-2 family members within a cell determines cell survival or death following damaging stimuli (Ouyang and Giffard, 2004). Bcl-xL is localized primarily to the outer mitochondrial membrane, where it functions to inhibit the mitochondrial permeability and...
serves to modulate cytochrome c release, free radical production, and calcium accumulation. Bcl-xL also blocks the effect of proapoptotic Bcl-2 family members by heterodimerization and regulation of the voltage-dependent anion channel on the outer membrane of mitochondria (Tsujimoto and Shimizu, 2000b) and the adenine nucleotide translocator on the inner mitochondrial membrane (Vieira et al., 2000). Recent reports document the capacity of Bcl-xL to block cell death after cerebral ischemia (Asoh et al., 2002). Proapoptotic Bak is localized predominantly in the cytosol of normal cells and translocates to mitochondria after apoptotic stimulation. Bak promotes cytochrome c release from mitochondria, leading to activation of caspase-9 and then caspase-3, which begins the degradation phase of apoptosis (Gauck and Eastman, 2002; Scorrano et al., 2003; Kleeh and Pimentel-Munoz, 2005).

In the auditory system, the localization of mRNAs of the Bcl-2 family proteins has been investigated in mouse embryos, in early postnatal development, and in adult mice (Ishii et al., 1996). Recent investigations have demonstrated a relationship between Bcl-2 family proteins and several cochlear disorders, such as increased sensitivity to neomycin-induced hearing loss (Cunningham et al., 2004), NIHL following sound conditioning (Niu et al., 2003), age-related hearing loss (Alam et al., 2004a), and cisplatin-induced hair cell death (Alam et al., 2000).

NIHL may be divided into two categories: temporary threshold shift (TTS) and permanent threshold shift (PTS). The former is associated with little or no sensory cell death, the latter with clear loss of sensory cells. This allows us to question the relative role of Bcl-2 genes in cells placed under extreme noise-induced metabolic stress sufficient to induce formation of free radicals; in one case, sensory cells recover and survive, whereas, in the other case, cell death follows. In this study, we investigated differences in the pathogenesis of TTS and PTS hearing loss by assessing the immunohistochemistry of Bcl-xL and Bak following exposure to sound levels that would produce TTS (cell survival) and PTS (cell death). We hypothesized that both genes would be expressed following these two stresses but that the balance of expression would differ significantly, with major expression of anti-apoptotic Bcl-xL in hair cells following exposure to TTS noise and proapoptotic Bak demonstrating major expression in hair cells following PTS exposure.

MATERIALS AND METHODS

Animals

Thirty-five pigmented guinea pigs (250–300 g; Elm Hill Breeding Labs, Chelmsford, MA) with a normal Preyer’s reflex were used in this study. The experimental protocol was reviewed and approved by the Animal Care and Use Committee at the University of Michigan and conforms to the National Institutes of Health Guide for the care and use of laboratory animals.

Noise Exposure

Animals were exposed in pairs, in separate cages, to one-octave-band noise centered at 4 kHz, at 100 dB SPL for 1 hr (TTS model) or at 120 dB SPL for 5 hr (PTS model), in a ventilated sound-exposure chamber. The sound chamber was fitted with speakers (model 2450H, JBL) driven by a noise generator (ME 604, graphic equalizer; Rane) and power amplifier (HCA-1000 high current power amplifier; Parasound Products). Sound levels were calibrated (Bruel and Kjaer Instruments, type 2203 precision sound level meter, type 4134 microphone) at multiple locations within the sound chamber to ensure uniformity of the stimulus and analyzed by using a fast Fourier transform network analyzer with a linear scale. The stimulus intensity varied by a maximum of 3 dB across measured sites within the exposure chamber. During noise exposure, noise levels were monitored with a sound level meter, a preamplifier, and a condenser microphone. The microphone was positioned within the cage at the approximate level of the animal’s head.

Auditory Brainstem Responses

Auditory evoked brainstem responses (ABR) were measured for both ears of each animal before and 1, 3, 7, and 10 days after noise exposure (10 animals, n = 5 at each exposure level). Prior to measurements, animals were anesthetized with intramuscular xylazine (10 mg/kg) and ketamine (40 mg/kg), and the external ear canal and tympanic membrane were inspected under an operating microscope (Yamashita et al., 2004a). Recordings were made from differential active needle electrodes placed subcutaneously below the test ear, at the vertex, and below the contralateral ear. The sound stimulus consisted of 15-msec tone bursts, with a rise-fall time of 1 msec, at frequencies of 4, 8, and 16 kHz and were generated by using a Fordham Audio Generator (model AG-298; Fordham Radio Supply, Hauppauge, NY). The stimuli were presented to the external auditory meatus via a closed acoustic system through a tube connected to a transducer (Beyer DT-48; Beyer Dynamic, Farmingdale, NY). The sound source was calibrated by coupling with a B&K 1/4-in. microphone through a 0.6-cc rubber tube. Starting levels of 100–105 dB SPL sound intensities were decreased in 10–20 dB steps to near-threshold levels and then in 5-dB decrements to define threshold. One thousand twenty-four tone presentations, delivered at 10/sec, were averaged to obtain a waveform, using a Tucker-Davis data acquisition system and a microcomputer with custom-written software. Threshold, tested separately for each ear, was defined as the lowest intensity of stimulation that yielded a repeatable waveform based on an identifiable ABR wave III or IV, whichever demonstrated greater sensitivity. Typically, ABR wave III was the most robust component of the guinea pig waveform, consistent with the finding of Puel et al. (1995). Thresholds were averaged across ears for each frequency in each animal.

Histological Assessment of Noise-Induced Hair Cell Pathology

Animals (n = 20) were euthanized under deep xylazine and ketamine anesthesia for histological study. Ten animals
In preliminary studies, Bcl-2, Bcl-xL, Bad, and Bak were assessed in immediately following exposure in which ABR was not assessed and another 10 animals at 10 days following exposure and the final ABR. After decapitation, the temporal bones were immediately removed and transferred into 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Under a dissecting microscope, the round and oval windows and the cochlear capsule near the apex were opened, followed by gentle local perfusion from the apex. The tissue was kept in fixative for 12 hr. After removal of the bony capsule and the lateral wall tissues, the modiolar core, including the organ of Corti, was removed from the temporal bone. After permeabilization with 0.3% Triton X-100 for 5 min, the organ of Corti was stained for f-actin with 1% rhodamine phalloidin for 40 min to outline hair cells and their stereocilia for a quantitative assessment of sensory cell loss (Raphael and Altschuler, 1991). After several washes with PBS, the organ of Corti was dissected and mounted as surface preparations. The tissues were observed under fluorescence microscopy, and the numbers of missing inner hair cells (IHC) and outer hair cells (OHC) were counted from the apex to the base in 0.19-mm segments, as described by Yamashita et al. (2004a). Counting was begun at 2.09 mm from the apex, omitting the initial irregular, most apical part of the cochlear spiral. Percentages of hair cell loss in each 0.19-mm length of tissue were plotted along the cochlear length as a cytocochleogram.

Immunohistochemistry for Bcl-xL, Bak

Immunocytochemistry was carried out in 15 animals (unexposed controls and day 1 after noise exposure at the two exposure levels, n = 5 each). The temporal bones were removed, transferred into 4% paraformaldehyde in PBS, locally perfused as described above, and kept in fixative overnight. After PBS rinses and removal of the bony capsule and the lateral wall tissues, the modiolar core, including the organ of Corti, was removed from the temporal bone. Tissue was incubated in blocking solution consisting of 3% normal goat serum (Anti-Bodies Incorporated, Davis, CA) in 0.3% Triton X-100 in PBS for 30 min. The organ of Corti was stained with Bcl-xL antibody (1:100; Cell Signaling Technology, Beverly, MA), or Bak antibody (1:100; Cell Signaling Technology) overnight at 4°C. Samples were washed three times in PBS and incubated for 1 hr in a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR). Tissues were then washed three times with PBS and incubated with a 1:100 dilution of rhodamine phalloidin (Molecular Probes) for 40 min to outline hair cells and their stereocilia. After three PBS rinses, tissues were incubated with 5 μg/ml propidium iodide (PI; Molecular Probes) in PBS for 15 min to mark the nuclei of dying OHC. After three PBS rinses, the organ of Corti was dissected and mounted as a surface preparation. Immunolabeling was visualized with an Olympus FV-500 confocal microscope. Photomicrographs representative of the 4-kHz region of the cochlea (primarily affected by the noise exposure) were selected from all animals. Those from each subject were then ordered based on background immunostaining (from weak to intense), and the middle photomicrograph was selected as representative for a given subject. These representative micrographs were then ordered, within each group, on the basis of background immunostaining intensity, and the photomicrograph at the midpoint of each of these groupings was selected as representative for each group of subjects. These midpoint images for each group were used to create the figures. The number of all positive immunoreactive cells for all subjects was counted from apical to basal turn and analyzed. Image scoring was conducted by an observer blind to the treatment protocol.

Statistical Analysis

All values presented in Results are mean ± SD. Differences among the different groups were evaluated by using one-way ANOVA. The Student-Newman-Keul post hoc test was then used to evaluate the significance of specific pairs of values.

RESULTS

ABR Threshold Shifts: Functional Assessment

Figure 1A,B illustrates the ABR threshold shifts observed following noise exposure at each exposure level, over time, through 10 days. Preexposure baseline ABR thresholds did not differ between groups and were consistent with normative data previously obtained in our laboratory (not shown).

After noise exposure, maximum threshold shifts were observed on day 1 in both groups. For animals exposed to the lower intensity level (100 dB SPL, 1 hr), maximum threshold shifts were 27.7 dB @ 4 kHz, 37.4 dB @ 8 kHz, and 11.4 dB @ 16 kHz. These threshold shifts gradually decreased, and completely recovered following days 7–10 for all frequencies. Thus these animals demonstrated a TTS. Animals exposed at the higher level (120 dB SPL, 5 hr) demonstrated high initial ABR threshold shifts of 40.0 dB @ 4 kHz, 59.8 dB @ 8 kHz, and 64.8 dB @ 16 kHz and stabilized following days 7–10 with a remaining threshold shift at all frequencies (22.6 dB @ 4 kHz, 42.5 dB @ 8 kHz, and 44.9 dB @ 16 kHz), similar to previous findings (Yamashita et al., 2004a). Thus, the lower noise exposure yielded a TTS model, whereas the higher level exposure yielded a PTS model of noise-exposure damage.

Hair Cell Loss: Morphological Assessment

As illustrated (Fig. 2A,B), in the animals of the PTS group (100 dB SPL, 1 hr), there was no significant loss of IHCs or OHCs either immediately or 10 days after noise exposure, including the 4-kHz region of the cochlea (approximately 10–12 mm from the apex). However, Figure 2C,D shows, in the animals of the PTS group (120 dB SPL, 5 hr) immediately following
exposure, a clear but restricted loss of only OHCs observed in the 4-kHz region of the cochlea (Fig. 2C).

Consistent with the observed PTS, by day 10 following exposure (Fig. 2D), a restricted IHC loss was found in the cochlear region 10–12 mm from the apex, and OHC loss had increased in both severity and extent (apically and basally) along the basilar membrane. The damage apical to the 6–10-mm region was due mostly to loss of OHCs in rows 2 and 3; more basally, damage was seen in all three rows. These data were consistent with previous observations (Yamashita et al., 2004a).

**Immunoreactivity to Bcl-xL and Bak**

Figures 3 and 4 were taken from the 4-kHz region (10–12 mm from apex) representing the focus of the most heavily damaged area. The numbers of positive immunoreactive cells were counted for all turns. Staining was absent from negative control material tested without the primary antibody (not shown).

In control animals and those exposed to the TTS noise (Fig. 3A,C), normal rhodamine phalloidin staining (blue) is seen in the stereocilia and cuticular plate at the surface of the organ of Corti. In the PTS-exposed animals (Fig. 3E,F), at several points, there is both absence of rhodamine phalloidin staining (blue) and PI staining (red) indicating the loss of hair cell sterecilia, cuticular plate, and their nuclei.

**Bcl-xL.** After the lower intensity TTS exposure (100 dB SPL, 1 hr), on day 1 after noise exposure, Bcl-xL-immunolabelled (green) OHCs were observed in row 1 at the depth of the nuclear region, consistent with cytosolic expression (Fig. 3D). Abundant immunoreactive staining was seen in both the 4-kHz region and some in the apical region. Little Bcl-xL staining was seen in control material or in the PTS model at any site throughout the cochlea.

**Bak.** As shown in Figure 4F, after the more intense exposure in the PTS group, marked Bak staining (green) was observed in the cytosol of OHCs at the depth of the nuclear region, mainly restricted to row 1. Some Bcl-xL staining was also observed. However, in contrast to the pattern of Bcl-xL staining, positive Bak staining was restricted to the immediate region of the nucleus and limited to the 4-kHz region. In controls and the TTS-exposure group, little Bak staining was seen.

The numbers of all positive Bcl-xL- and Bak-immunoreactive cells for all subjects were counted from apex to basal turn. The mean across subjects of each experimental group was determined for each row of hair cells. As shown in Figure 5A, Bcl-xL labeling is significantly greater in the TTS model than in control and PTS models ($P < 0.01$). Bak-immunostained cells were significantly greater in number in the PTS model than in the control and TTS-exposed material (Fig. 5B; $P < 0.01$).

**DISCUSSION**

In this study, we demonstrated the expression of the antiapoptotic factor Bcl-xL and the proapoptotic factor Bak following exposure to high levels of noise stimulation. However, differential expression of these genes was specifically dependent on the noise exposure intensity-duration. Bcl-xL was expressed following exposure to a sound that causes no sensory cell loss and yields a temporary change in hearing threshold with complete recovery (TTS model). Bak was expressed in response to a more intense sound that causes a permanent change in the threshold and sensory cell death (PTS model). The expression of these gene products was observed at that region of the cochlea most sensitive to the major spectral components of the sound exposure (the 4-kHz region), localized approximately 10–12 mm from apex. These results indicate that Bcl-xL may act to suppress cochlear
cell death induced by TTS-level noise exposure, whereas
the expression of Bak, in the PTS model, may be a
causative factor in induction of OHC apoptosis. These
findings indicate a major role for Bcl-2 family proteins
in NIHL and the survival or death of sensory hair cells
following exposure to high levels of noise.

The temporary threshold shifts after noise exposure
in both the TTS and PTS models are thought to arise
from various reversible structural or functional changes
in the cochlea, such as changes in the mechanical com-
pliance of the basilar membrane (LePage, 1989),
decreased stiffness of the hair cell stereocilia (Saunders
et al., 1986), and afferent terminal excitotoxicity (Pujol
and Puel, 1999). Any of these factors, if sufficiently
intense, may lead to sensory cell death. Presumably, this
depends on the level of noise and damage induced dur-
ing or shortly following noise exposure, undoubtedly
with a number of factors determining whether reversible
or irreversible alterations occur. The results of this study
suggest that, in the TTS model, expressions of Bcl-xL
may be a factor protecting damaged hair cells from death
following exposure. One mechanism by which Bcl-xL

Fig. 2. Cytocochleograms, for each experimental group and time point, show the average percent-
age hair cell loss (IHC and OHCs row 1–3) of each group (n = 5). In the TTS model (A,B), no
significant loss of IHC and OHCs is seen either immediately or on day 10 after noise exposure. In
the PTS model, limited damage, located approximately 10–12 mm from the apex, is observed imme-
diately after intense noise (C), which then increased and spread apically and basally by day 10 (D).

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may mediate this survival is by blocking the release of
cytochrome c from the mitochondrial membrane into
the cytosol, reducing excess free radical formation and
allowing repair processes to affect a complete recovery.
However, Bak expressed in response to greater levels of
noise exposure with greater tissue effects may signifi-
cantly contribute to cell death by permeabilization of
the mitochondrial outer membrane and release of proteins
such as cytochrome c that initiate apoptosis.

Bcl-xL and Bak expression was primarily restricted
to row-1 OHC, with Bcl-xL expression extending to
more apical region. Morphologically, we observed that
the damage apical to the 4–8-kHz region (i.e., 6–10 mm
from apex) reflected OHC cell death in rows 2 and 3,
whereas, toward the base, damage was seen in all three
rows. Because of the timing of loss of OHC (row 2 and
3 following row 1), we previously hypothesized that api-
cal row 2 and 3 cell death reflects mainly necrosis
induced secondary to apoptotic events in row 1 OHC
(Yamashita et al., 2004a). Given Hu et al.’s (2002) report
that, 2 days after noise exposure, the numbers of frag-
mented and condensed (apoptotic) nuclei are dramati-
cally shifted from the 4-kHz region toward the basal
part of the cochlea, we suggest that it is the apical
expression of Bcl-xL, observed in this study, that signifi-
cantly limits the spread of cell death from the 4-kHz
region to lower (apical) regions of the cochlea, particu-
larly in row 1 OHC.

Support for the potential role of Bcl-2 proteins in
noise-induced cell survival and death is also based on
previously reported findings that calcineurin activity is
up-regulated in hair cells 24 hr after noise exposure
(Minami et al., 2004). Calcineurin expression, which is
controlled by \([\text{Ca}^{2+}]\), and calmodulin, activates cell

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Fig. 3. Surface preparation of cochlear tissue taken day 1 after noise exposure and triply labeled for
Bcl-xL (ant apoptotic; green), phalloidin (blue), and PI (red) shown at two depths from the surface
of the organ of Corti: cuticular plate (A,C,E) and nuclear region (B,D,F). Control results (A,B)
and TTS (C,D) and PTS (E,F) results are shown. Bcl-xL immunoreactivity at the nuclear region of
OHC row 1 is seen exclusively in the TTS model (D).
death pathways by dephosphorylating the Bcl-2 family protein Bad (Wang et al., 1999) and inactivating the antiapoptotic Bcl-2 (Erin et al., 2003). Other studies have shown that, with the permeabilization of the outer mitochondrial membrane by the activation, translocation, and oligomerization of multidomain Bcl-2 family proteins, EndoG, an apoptotic nuclease belonging to a family of Mg\(^{2+}\)-dependent nucleases (Miller et al., 1999), is released (Arnoult et al., 2003; Er et al., 2006). Recently Yamashita et al. (2004b) demonstrated that EndoG is expressed in the inner ear 24 hr following noise exposure and translocates to the nucleus after noise exposure, where it induced caspase-independent apoptotic cell death. These new data are consistent with a key role of Bcl-2 genes in the response of the inner ear to acoustic trauma.

The findings of this paper support many recent studies showing that overexpression of Bcl-2 family proteins can reduce neuronal cell death, including ischemic brain injury, in animal models. Defective herpes simplex viral (HSV) vectors were utilized to induce Bcl-2 overexpression, which was protective against excitotoxic insults in vitro and in vivo (Lawrence et al., 1996). Bcl-2-based gene therapy was effective even when given after the onset of injury (Dumas et al., 2000), and Zhao et al. (2003) demonstrated Bcl-2 gene transfer to the margin of infarcts, providing protection from ischemia. This protection was accompanied by reduced cytochrome c translocation to the cytosol and reduced activation of caspase-3. Finally, Cao et al. (2002) reported that Bcl-xL inhibited staurosporine-induced apoptosis in primary cultured neocortical neurons and reduced neu-
ronal death resulting from transient focal ischemia in the intact brain.

Gene and protein therapy using antiapoptotic Bcl-2 family members may provide significant protection against neural injury, including sensory cells of the inner ear in association with NIHL. We have recently demonstrated that both pre- and postnoise exposure treatment with a combination of antioxidants (Yamashita et al., 2005) can be effective in significantly reducing NIHL. This strategy holds promise for reducing this important and costly disorder. However, with recent work showing a caspase-independent, apoptotic, EndoG-driven cell death pathway in NIHL (Yamashita et al., 2004b), and a role for Bcl-2 family genes in modulating noise-induced hair cell loss (which may, in part, reflect a noise-induced calcineurin up-regulation), it is clear that many aspects of the biochemical pathways involved in NIHL remain to be identified and analyzed. With each additional insight, we should define new and improved interventions that may be translated from the laboratory to clinical application and, with that, significantly reduce this important cause of hearing impairment.

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