Nuclear Factor kappa B p65 Expression in Mouse Cochlea

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Abstract
Nuclear factor kappa B (NF-κB) is one of the best-characterized transcription factors playing important roles in many cellular responses to a large variety of stimuli, including inflammatory cytokines, phorbol esters, growth factors, and bacterial and viral products. The aim of this study is to demonstrate NF-κB expression in the mouse cochlea and its enhancement in response to lipopolysaccharides (LPS) and kanamycin (KA) treatment.

Methods
KA treatment consisted of subcutaneous KA injections at 700 mg/kg twice a day with an eight-hour interval between the two injections for 3 or 7 days. For animals in the LPS treatment group, a single dose of 0.3 mg LPS dissolved in 0.2 ml sterile saline were injected into both bullae through the tympanic membrane and kept there for 3 hours. Animals in the control group received subcutaneous saline injection for 7 days. Following immunohistochemical processing with rabbit polyclonal anti-NF-κB p65 antibodies, cryosections of the cochlea were examined for expression of NF-κB p65 in various structures in the cochlea.

Results
NF-κB p65 expression, identified by presence of brown reaction products characteristic of DAB immunohistochemistry, was visible in the spiral ligament, spiral prominence, tectorial membrane (TM), spiral ganglion and nerve fibers. Relatively weak NF-κB p65 expression was also visualized in the organ of Corti. Within the organ of Corti, the inner hair cells (IHC), outer hair cells (OHC), inner pillar cells (IP), outer pillar cells (OP), Deiter’s cells (DC), and Böettcher’s cells exhibited stronger staining than the inner sulcus cells, Hensen’s cells (HC) and Claudius’ cells. No NF-κB p65 expression was seen in the nucleus of the IHC and OHC. NF-κB p65 expression was increased in animals exposed to LPS or KA, demonstrating significant differences in the staining between control animals and LPS/KA-treated animals. NF-κB p65 expression was not significantly different between LPS treated and KA treated animals or between 3 and 7 days in KA-treated animals.

Conclusion
LPS and KA exposure increases expression of NF-κB p65 in the mouse cochlea.

Key words
transcription factors; nuclear factor kappa B p65 (NF-κB p65); mouse cochlea; immunohistochemistry; lipopolysaccharide (LPS)

Introductions
NF-κB is one of the most important transcription factor playing a pivotal role in many cellular responses to a wide variety of signals, including inflammatory cytokines, phorbol esters, growth factors, bacterial and viral products, oxidative stress, hypoxia/reoxygenation, UV light and radio irradiation. NF-κB is present in the cytoplasm of the majority of cell types. It serves as a critical regulator of the inducible expression of genes involved in processes of immunity and inflammation, cell adhesion, as well as in cell growth and death [1]. NF-κB was first described as a protein that binds specifically to a DNA sequence (5’-GGGACTTTCC-3’) in the intronic enhancer of the immunoglobulin k light chain gene [2]. NF-κB is a member of the larger NF-κB/Rel family of transcriptional factors, which includes p50 (NF-κB 1), p52 (NF-κB 2), p65 (Rel A), RelB, and C-Rel.
NF-κB/Rel proteins exist as homo- or heterodimers and are sequestered in an inactive form through non-covalent interaction with an inhibitory protein referred to as IκB. To date, some members of the IκB family have been identified, all of them (IκB–α, IκB–b, IκB–γ, IκB–e, Bcl–3, p100, and p105) containing multiple copies of a 30–33 amino acid sequence called ankyrin repeats. The specific interaction between ankyrin repeats and the Rel-homology domain of NF-κB/Rel proteins prevents the nuclear translocation of the NF-κB/IκB complex. When cells are appropriately stimulated with NF-κB inducers, the IκB kinase (IKK) complex is activated, IκB is phosphorylated at positions Ser32 and Ser36 (phosphorylations of other serines as well as tyrosines and threonines have been also reported, but their precise role is unclear at the moment), and then ubiquitinated on Lys22. These events trigger the rapid degradation of IκB by the 26s protease, thereby releasing active NF-κB. Finally, the released NF-κB dimer translocates into the nucleus, where it binds DNA and induces transcription of specific target genes.

The current study is aimed at demonstrating the location of the NF-κB p65 proteins in the mouse cochlea and studying the effects by KA and LPS treatment on their expression.

Materials and methods

Experimental animals

Twenty four mice (20–25g), purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), were randomly divided into four groups: a KA 3 day treatment group, a KA 7 day treatment group, a LPS treatment group and a control group. There were 6 animals in each group. The animals had free access to water and a regular mouse diet (Purina 5025, St. Louis, MO), and were allowed 1 week of adaptation. Animal care was under the supervision by the University of Michigan’s Unit for Laboratory Animal Medicine (ULAM). The Committee on Use and Care of Animals approved all test protocols.

Drug administration

KA sulfate was purchased from USBTM Corporation (Cleveland, OH), and LPS from Sigma Chemical Co. (St. Louis, MO). KA was dissolved in saline at a concentration of 44.7 mg/ml and was subcutaneously injected at 700 mg/kg twice a day with an eight hours interval between the two injections. KA was administrated for 3 or 7 days before harvesting the temporal bone for the two groups of animals receiving KA treatment, respectively. For animals in the LPS treatment group, a single dose of 0.3 mg LPS dissolved in 0.2 ml sterile saline was injected into both bullae through the tympanic membrane and kept ther for 3 hours. Animals in the control group received subcutaneous saline injections for 7 days.

Tissue preparation

Animals were sacrificed via neck dislocation and the bullae rapidly opened. Stapes were removed. The round window membrane and cochlear tips were perforated with a fine needle. The cochlea was gently perfused with 4% paraformaldehyde fixative in 10 mM PBS (pH 7.4). Temporal bones were immersed in the fixative for overnight and rinsed in 10 mM PBS twice. Cochleae were decalcified in 8% EDTA solution at 4°C for a total of 1.5 days, followed by rinsing in PBS. The cochleae were then washed for 30 minutes in 10% sucrose, 2:1 solution of 10:30% sucrose, 1:1 solution of 10:30 % sucrose, 1:2 solution of 10:30 % sucrose at room temperature with rotation, and maintained overnight at 4 °C in 30% sucrose. The cochleae were placed in the cryomold under dissecting microscope and filled halfway with OCT. Embedded in OCT and oriented by aligning an imaginary plane through the modiolus parallel with the bottom of the embedding mold, the mold was immediately placed in the dry ice/ethanol bath. Five-micron sections were cut using a Leica Cryomicrotomed 3000. Sections were mounted on Fisherbrand superfrt plus slides and stored in the refrigerator at 4°C.

Immunohistochemical Staining

Immunohistochemical procedures have been described in detail in other studies. Sections were immersed at room temperature first in 0.3% Triton X 100 for 10 minutes and then in 0.3% hydrogen peroxide for 30 minutes. Sections were then rinsed twice in 10 mM PBS. This was followed by incubation with diluted normal blocking serum (Vector Elite ABC kit) for 30 minutes and with 1:24000 rabbit polyclonal antι-NFκB-p65 (primary) antibodies (Santa Cruz Biotechnology) for 30 minutes at room temperature. Biotinylated secondary antibody solution and vectastain Elite ABC reagent were applied for 30 minutes. The sections were rinsed again with PBS and allowed to react in substrate medium containing 3’ 3-diaminobenzidine (DAB; sigma) and hydrogen peroxide for 3 minutes before observation under light microscope. Incubation with primary antibodies was omitted for control sections.
Results

NF-κB P65 expression was identified by presence of brown reaction products characteristic of DAB immunohistochemistry. Expression was graded based upon the density of the reaction products, from light brown (+), to moderate brown (++) and to dark brown (+++), with darker staining representing stronger expression. Lack of reaction product staining was graded with “−”, as seen in control sections that were not exposed to the primary antibodies (see Table 1).

Expression of NF-κB P65 in the cochlea in control animals

In control animals, NF-κB p65 expression was represented mainly by light brown(+) staining. The staining was seen in the organ of Corti, spiral limbus, tectorial membrane (TM), spiral ligament, spiral prominence, spiral ganglion and nerve fibers in all turns throughout the cochlea. Of these structures, staining in the organ of Corti was relatively weak compared to others. Within the organ of Corti, NF-κB p65 expression was observed in the plasma of OHC and IHC, HC, IP and OP, DC and TM, but not in the nucleus, with relatively weak staining in inner sulcus cells, Hensen’s cells and Claudius’ cells. There was no NF-κB p65 expression in the basilar membrane (Figure 1) or the stria vascularis of normal group (Figure 2). In the spiral ganglion, expression of NF-κB

Table 1. Strength of NF-κB p65 expression in various structures in the mouse cochlea

<table>
<thead>
<tr>
<th>Tissues and cells</th>
<th>Control</th>
<th>LPS</th>
<th>KA 3 day treatment</th>
<th>KA 7 day treatment</th>
</tr>
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<tr>
<td>IHC plasm (nucleus)</td>
<td>(+/-)</td>
<td>++(-)</td>
<td>+++(+)</td>
<td>++++(+)</td>
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<tr>
<td>OHC plasm (nucleus)</td>
<td>(+/-)</td>
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<td>Inner pillar plasm (nucleus)</td>
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<td>++++(+)</td>
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<tr>
<td>Outer pillar plasm (nucleus)</td>
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<td>++(-)</td>
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<td>Deiters cell plasm (nucleus)</td>
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<td>Hensen cell plasm (nucleus)</td>
<td>(+/-)</td>
<td>++(-)</td>
<td>+++(+)</td>
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<tr>
<td>Boettcher cell plasm (nucleus)</td>
<td>(+/-)</td>
<td>++(-)</td>
<td>+++(+)</td>
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<tr>
<td>Inner sulcus plasm (nucleus)</td>
<td>(+/-)</td>
<td>++(-)</td>
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<td>++++(+)</td>
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<tr>
<td>Claudius cell plasm (nucleus)</td>
<td>(+/-)</td>
<td>++(-)</td>
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<tr>
<td>Tectorial membrane</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Stria vascularis</td>
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<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Fiber of spiral nerve</td>
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<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Basal membrane</td>
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Note: NF-κB P65 expression was identified by presence of brown reaction products characteristic of DAB immunohistochemistry. Expression was graded based upon the density of the reaction products, + presents light brown, ++ is moderate brown and +++ is dark brown with darker staining representing stronger expression. Lack of reaction product staining was expressed with “−”
p65 was present in the plasma, nucleus and nerve fibers (Figure 3).

**NF-κB P65 expression in LPS treated animals**

Increased expression of NF-κB p65 was seen in the plasma of OHC and IHC, HC, IP and OP, DC and TM with darker staining, compared to control animals. Again, there was no NF-κB p65 expression in the nucleus of these cells or in the basilar membrane (Figure 4). Increased levels of NF-κB p65 expression was also seen in the spiral prominence (Figure 5), and in the plasma, nucleus and nerve fibers of the spiral ganglion (Figure 6). Low level NF-κB p65 expression was present in the spiral ligament in this group of animals.

**NF-κB p65 expression in KA treated animals**

In the two groups of animals treated with KA, expression of NF-κB P65 in the plasma of OHC and IHC, HC, OP and IP, DC and TM in the cochlea from a KA treated animal appeared to be further increased, compared to both control and LPS treated animals. The nucleus of these cells, however, again showed no NF-κB p65 expression. Also, there was no staining in the basilar membrane (Figure 7). The stria vascularis showed light staining as in LPS treated animals. Much increased NF-κB p65 expression was also observed in the spiral ligament,
spiral prominence (Figure 8), and in the plasma, nucleus and nerve fiber of the spiral ganglion (Figure 9), compared to the control and LPS treated animals.

The differences between control and LPS/KA-treated animals concerning NF-κB p65 expression were statistically significant. There was no statistically significant difference in NF-κB p65 expression between 3 and 7 days of KA treatment.

**Discussion**

NF-κB is a member of the Rel family of transcriptional activator proteins and has a diverse variety of actions in the central nervous system. NF-κB has been found to regulate many genes that have important functions in conditions such as inflammation and immunity [1]. All members of the Rel family of transcription factors share sequence homology. The subunits can dimerize to be homodimers or heterodimers although NF-κB usually exists predominantly as a heterodimer of p50 and p65. There are three forms of NF-κB present in neurons in vivo, namely, constitutive NF-κB activity in the nucleus, free inhibitory protein IκB, and inactive NF-κB in the cytoplasm and synapse closely bound to the inhibitory protein IκB, which keeps it inactive. Several extracellular stimuli, including cytokines, viruses, bacterial LPS, NO, and oxidative stress, can cause the phosphorylation and consequent ubiquitination of the IκB protein, resulting in the translocation of active NF-κB from the cytoplasm to the nucleus. Activated NF-κB binds DNA in the nucleus and hence regulates gene expression. Several genes have NF-κB binding sites, including enzymes (such as the inducible nitric oxide synthase [iNOS] and cyclooxygenase isoform 2), neuropeptides (such as dynorphin and proenkephalin), and adhesion molecules (such as cell adhesion molecule-1 and intercellular adhesion molecule-1).

The current study demonstrated positive expression of NF-κB p65 in a variety of structures in the normal cochlea, including many types of cells in the organ of Corti, the spiral limbus, TM, spiral ligament, spiral ganglion and nerve fibers. This indicates the existence of NF-κB in these cells and tissues, likely the inhibitory IκB with an inactive form. Our study showed no NF-κB p65 expressions in the nucleus of cells in the organ of Corti, nor in the basal membrane and stria vascularis. Positive NF-κB p65 expression was also present in the plasma and nucleus of the spiral ganglion. Baldwin reported that NF-κB usually exists in an inactive form, (i.e., p50 and p65 dimer combined with the inhibitory IκB) in the resting condition [7]. It is not clear if the NF-κB p65 expression in the spiral ganglion nucleus observed in this study represents active NF-κB p65 activity.

NF-κB p65 expression in the above mentioned structures appeared to have been enhanced following exposure to LPS, suggesting activation of NF-κB p65 by LPS stimulation, which induces transduction of NF-κB from the plasma to the nucleus. Inside the nucleus, NF-κB will be activated through combining with DNA and regulate gene expression. The increased NF-κB p65 expression in the cochlea in response to middle ear exposure to LPS indicates an inflammatory reaction in the cochlea induced by presence of stimulating factors in the middle ear.

Jiang et al reported progressive destruction of hair cells by kanamycin, with largely preserved auditory function and structures after 7 days of treatment, which allowed investigation of early events in drug-induced cell death. NF-κB expression increased at 3 hr, 3 days, and 7 days following kanamycin treatment, with no labeling in nuclei of outer hair cells. Concomitant injections of antioxidants, 2,3-dihydroxybenzoic acid or salicylate (which prevents cell death induced by kanamycin), promoted translocation of NF-κB into the nuclei of outer hair cells [8]. Our results seem to agree with those in Jiang’s study. Significantly increased NF-κB p65 expression was seen in animals receiving systemic KA, suggesting its strong effects on NF-κB p65 in the cochlea. Masatsugu also showed in an immunohistochemical study that there was nuclear translocation of both p65 and p50 in the lateral wall after noise exposure. These results seem to be a close association between p65 and p50. Expression of inducible nitric oxide synthase was also enhanced by noise exposure. Their results suggest that NF-κB may
have a detrimental role in the response to acoustic overstimulation in the cochlea of mice \(^9\). As an antibiotic, KA does not induce inflammation in the cochlea. We speculate that the strong NF–κB p65 expression seen in this study may represent an immune response in the cochlea as part of the body’s response to KA exposure. This immune response may also cause damage in various structures in the cochlea. Whether KA can cause cochlear damage by directly activating NF–κB p65 needs to be further studied. An increased understanding of this issue will likely provide new knowledge in KA ototoxicity and hopefully lead to improved prevention and treatment.

References