

# The Influence of NF- $\kappa$ B Signal-transduction Pathways on the Murine Inner Ear by Acoustic Overstimulation

Hiroshi Yamamoto,<sup>1,2</sup> Irina Omelchenko,<sup>1</sup> Xiaorui Shi,<sup>1,3</sup> and Alfred L. Nuttall<sup>1,3,4\*</sup>

<sup>1</sup>Oregon Hearing Research Center, Oregon Health and Science University, Portland, Oregon

<sup>2</sup>Department of Otorhinolaryngology, Nagoya University School of Medicine, Nagoya, Japan

<sup>3</sup>Department of Otolaryngology, Renji Hospital, Shanghai Jiao Tong University, Shanghai, China

<sup>4</sup>Kresge Hearing Research Institute, University of Michigan, Ann Arbor, Michigan

Nuclear factor-kappa B (NF- $\kappa$ B) comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory responses. The NF- $\kappa$ B signals are activated via the canonical and/or noncanonical pathways in response to diverse stimuli. The excessive action of NF- $\kappa$ B signal-transduction pathways frequently causes self-injurious phenomena such as allergic diseases, vascular disorders, and ischemia-reperfusion neuronal damage. In the inner ear, the role of NF- $\kappa$ B has not been clarified because the activated NF- $\kappa$ B signals potentially induce both cytoprotective and cytotoxic target genes after ototoxic stimulation. In the present study, we investigated the response of NF- $\kappa$ B in both the canonical and noncanonical pathways to acoustic overstimulation (117 dB/SPL/2 hr) and followed the change of inflammatory factors (inducible nitric oxide synthase [iNOS], intracellular adhesion molecule-1 [ICAM-1], and vascular cell adhesion molecule-1 [VCAM-1]) in the cochlear lateral wall (CLW) and the rest of cochlea (RoC). By means of immunohistochemistry combined with confocal microscopy and reverse transcriptase-polymerase chain reaction techniques, we found the response of NF- $\kappa$ B family members (NF- $\kappa$ B1, 2, RelA, and RelB) at the transcription level. After the NF- $\kappa$ B signaling, the inflammatory factors were significantly increased in the CLW and the RoC. Additionally, at the protein level, the prominent expression of adhesion molecules (ICAM-1 and VCAM-1) was observed in the tissue around the capillaries in the stria vascularis. These results show that acoustic overstimulation causes the NF- $\kappa$ B signaling to overexpress the inflammatory factors in the inner ear, and the up-regulation of the adhesion molecules (ICAM-1 and VCAM-1) and iNOS potentially influence the hemodynamics and the cellular integrity in the stria vascularis. © 2009 Wiley-Liss, Inc.

**Key words:** stria vascularis; iNOS; ICAM-1; VCAM-1; noise exposure

The nuclear factor-kappa B (NF- $\kappa$ B)/Rel family is a group of structurally and evolutionally conserved pro-

teins, which is expressed in all cell types and functions as a sequence-specific transcription factor (Sen and Baltimore, 1986; Ghosh et al., 1998). Five members of the NF- $\kappa$ B/Rel family, NF- $\kappa$ B1 (p105/p50), NF- $\kappa$ B2 (p100/p52), RelA (p65), RelB, and c-Rel, are identified in mammalian cells and known to exist as homo- or heterodimeric complexes in the cytoplasm (Ghosh et al., 1998; Rothwarf and Karin, 1999). The NF- $\kappa$ B signals are activated through either or both of two independent signal-transduction (canonical and noncanonical) pathways in response to inflammation, infections, and other stressful situations requiring rapid reprogramming of gene expression (Ghosh et al., 1998; Rothwarf and Karin, 1999; Senftleben et al., 2001). Although various homo- and heterodimeric combinations possibly function in the NF- $\kappa$ B/Rel family, the heterodimers of NF- $\kappa$ B1 and RelA in the canonical pathway as well as the heterodimers of NF- $\kappa$ B2 and RelB in the noncanonical pathway are considered to be predominant respectively (Baeuerle and Henkel, 1994; Rothwarf and Karin, 1999; Siebenlist et al., 2005). NF- $\kappa$ B signals trigger the expression of various target genes encoding inducible effectors (e.g., inducible nitric oxide synthase [iNOS] and cyclooxygenase-2), adhesion molecules (e.g., intracellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]), cytokines, and chemokines, which play a critical role in innate and adaptive immune responses (Baeuerle and Henkel, 1994; Ghosh et al.,

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\*Correspondence to: Alfred L. Nuttall, Oregon Health and Science University, Oregon Hearing Research Center, 3181 SW Sam Jackson Park Road, NRC04, Portland, OR 97239-3098. E-mail: nuttall@ohsu.edu

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1998; Ghosh and Karin, 2002). Furthermore, the NF- $\kappa$ B/Rel family is involved in cell death, survival, proliferation, development, and degeneration by inducing antiapoptotic and proliferative factors (Beg et al., 1995; Denk et al., 2000; Karin and Lin, 2002). On the other hand, the overstimulation of NF- $\kappa$ B signals frequently causes cellular and DNA damage from reactive oxygen species (ROS) mediated by iNOS (Moncada et al., 1991; Cuzzocrea et al., 2001; Conti et al., 2007) and produces hemodynamic change associated with ICAM-1 and VCAM-1 (Collins et al., 1995; Sumagin and Sarelius, 2007). The overproductive and long-term activation can lead to vascular insufficiency such as cardiovascular disorders (Miwa et al., 1997; Ridker et al., 1998; Valen et al., 2001), allergic tracheobronchial lesions in asthma (Hart et al., 1998), and malignancy (Karin et al., 2002).

Several studies found that in the inner ear, the NF- $\kappa$ B/Rel family is potentially required for normal hair cell function including  $\text{Ca}^{2+}$  homeostasis (Nagy et al., 2005; Lang et al., 2006) and the signal-transduction pathways can rapidly respond to ototoxic stimulants, such as noise exposure and ototoxic drugs, to protect hair cells and spiral ganglion cells (Jiang et al., 2005; Nagashima et al., 2007). On the other hand, other studies suggested that NF- $\kappa$ B induced overexpression of iNOS is presumably involved in insults of the cochlear lateral wall (CLW) by producing large amounts of ROS (Watanabe et al., 2002; Shi and Nuttall, 2003; Masuda et al., 2006). Actually, the cytoprotective role of the NF- $\kappa$ B/Rel family in the murine inner ear is incomplete or inefficient to acoustic overstimulation (116 dB/SPL/2 hr) because a marked hair cell degeneration occurs (Hirose and Liberman, 2003). Thus, past studies revealed the noise-induced NF- $\kappa$ B activation and its potential roles for cytoprotection and cytotoxicity in the inner ear (Lang et al., 2006; Masuda et al., 2006). There is, however, little information regarding the deeper questions of which NF- $\kappa$ B signal-transduction pathway is involved and what target genes may affect cochlear function with acoustic overstimulation.

In the present study, we investigated the response of the NF- $\kappa$ B/Rel family members (NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, and RelB) in canonical and noncanonical pathways at the transcription level by acoustic overstimulation (117 dB/SPL/2 hr) and followed the time course of inflammatory factors (iNOS, ICAM-1, and VCAM-1) in the inner ear.

## MATERIALS AND METHODS

Male CBA mice aged 10–12 weeks with normal hearing were used. To determine the response of the NF- $\kappa$ B/Rel family members (NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, and RelB) in the whole cochlea, six animals were randomly assigned to serve as controls or as noise-exposed subjects and prepared for reverse transcriptase–polymerase chain reaction (RT-PCR). For quantitative real-time PCR analysis, 18 animals were divided into six groups: control group, 2-hr noise exposure (NE 2h) group, 2-hr waiting after the NE 2h (NE 2h+2h) group, 4-hr waiting after the NE 2h (NE 2h+4h) group, 6-hr waiting

after the NE 2h (NE 2h+6h) group, and 12-hr waiting after the NE 2h (NE 2h+12h) group. Each group comprised three animals. The noise exposure level was 117 dB/SPL/broadband for 2 hr. After rats were humanely killed with an overdose of ketamine hydrochloride (100 mg/kg, i.m.) and 2% xylazine hydrochloride (10 mg/kg) (Abbott Laboratories, N. Chicago, IL), the CLW tissues and the RoC tissues, which include the organ of Corti with supporting cells and spiral ganglion cells, were dissected and grouped from whole cochleas, and cDNA samples were prepared for RT-PCR and quantitative real-time PCR analysis. To evaluate the change of adhesion molecules (ICAM-1 and VCAM-1) in the CLW, four animals were divided into two groups, the control group and 14-hr waiting after the NE 2h (NE 2h+14h) group, and prepared for immunohistochemistry. The procedures of this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University.

## Immunohistochemistry

Cochleas were dissected from the control group and the NE 2h+14h group ( $n = 2$ , in each) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 hr, then the CLW were isolated and permeabilized with 0.5% Triton X-100 for 1 hr. After being immunoblocked with blocking solution consisting of 10% goat serum/2% bovine serum albumin (BSA) in PBS for 1 hr, the samples were incubated overnight at 4°C in the antibodies for ICAM-1 and VCAM-1 (rabbit polyclonal anti-ICAM-1, rabbit polyclonal anti-VCAM-1, diluted 1:100 with 5% BSA in PBS, Santa Cruz Biotechnology). The specimens were also double labeled with propidium iodide (PI; 500 nM, Molecular Probes/Invitrogen) for 30 min at room temperature. The specimens were rinsed several times with PBS and incubated with a secondary antibody (Alexa Fluor 488 goat anti-rabbit, diluted 1:100 with 5% BSA in PBS, Molecular Probes/Invitrogen) for 1 hr at room temperature. After rinsing, the tissues were mounted and observed on a Nikon Eclipse TE 300 inverted microscope fitted with a Bio-Rad MRC 1024 confocal laser microscope system. Negative control tissues were incubated with 5% BSA in PBS to replace the primary antibodies and double labeled with PI.

## Total RNA Extraction and cDNA Synthesis From Cochlear Tissues

Total RNA was extracted from the cochleas isolated from each group ( $n = 3$ ) divided into the CLW and the RoC by with RNeasy Micro Kit (Qiagen, Valencia, CA) following the standard protocol. First-strand cDNA of each sample was synthesized from 1–2  $\mu$ g of total RNA by with RETROscript Kit (Ambion). The reaction was finally heat-inactivated. Separately, the total RNA of whole cochleas was extracted from six ears of the control and noise-exposed (NE 2h+4h) subjects ( $n = 3$  in each), and the cDNA was prepared for RT-PCR by the same protocol.

## RT-PCR and Quantitative Real-time PCR Analysis

Conserved regions spanning introns were selected to design the primers for the NF- $\kappa$ B/Rel family: iNOS, adhesion molecules and glyceraldehyde-3-phosphate dehydrogenase

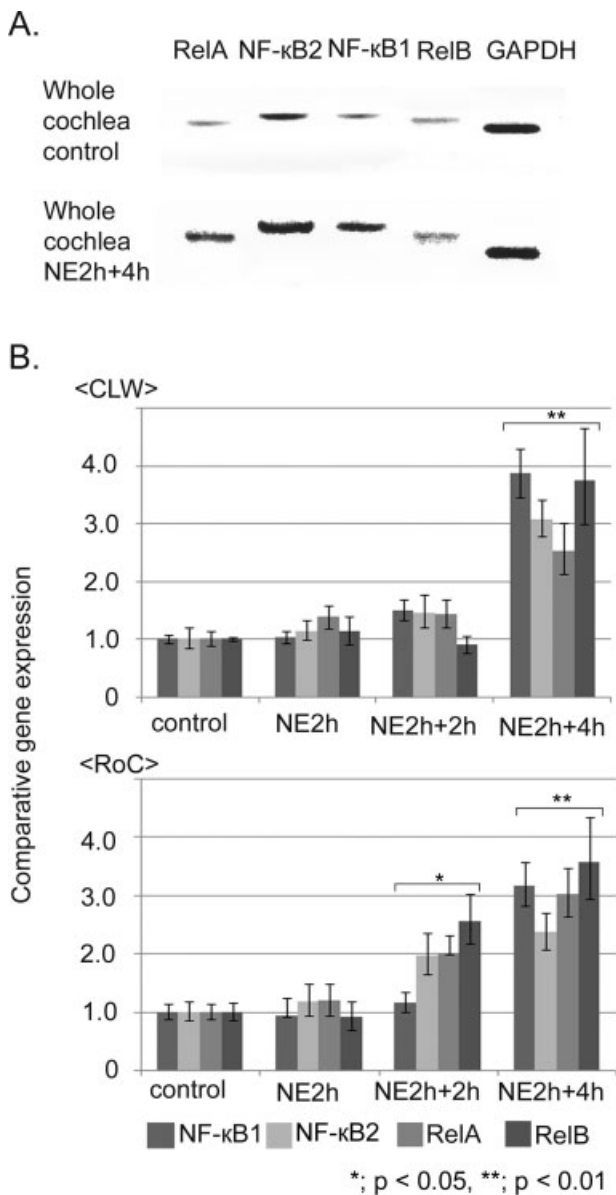


Fig. 1. Response of the NF- $\kappa$ B/Rel family members to acoustic overstimulation. **A:** PCR products of NF- $\kappa$ B1 (194 bp), NF- $\kappa$ B2 (195 bp), RelA (167 bp), and RelB (150 bp) in whole cochleas by RT-PCR. Their intensity at 30 cycles in the NE 2h+4h group is higher than those of the control group. GAPDH (132 bp) was used as a reference gene and compared at 24 cycles. **B:** Quantification of the NF- $\kappa$ B/Rel family members' expressions in the CLW and the RoC by quantitative real-time PCR analysis. Error bars represent SD. Each value estimated by the  $2^{-\Delta\Delta CT}$  method represents a comparison with the control group defined as 1.0. Statistical differences in the data sets between the control group and the NE groups were assessed with Student's *t*-tests and considered significant at \* $P < 0.05$  and at \*\* $P < 0.01$ . Statistics: control/NE 2h; CLW:  $P = 0.057$ , RoC:  $P = 0.271$ , control/NE 2h+2h; CLW:  $P = 0.054$ , RoC: \* $P = 0.025$ , control/NE 2h+4h; CLW: \*\* $P = 0.003$ , RoC: \*\* $P = 0.002$ .

(GAPDH). The primers used were as follows: NF- $\kappa$ B1 (mouse Chr 3 NM\_008689), forward, GAAATTCCTGATC CAGACAAAAAC, reverse, ATCACTTCAATGGCCTCT GTGTAG, 194-bp product; NF- $\kappa$ B2 (mouse Chr 19 NM\_019408), forward, CTGGTGGACACATACAGGAA GAC, reverse, ATAGGCACTGTCTTCTTTTCACCTC, 195-bp product; RelA (mouse Chr 19 NM\_009045), forward, CTCCTCAGCCATGGTACCTCT, reverse, CAAGTCTT CATCAGCATCAAACCTG, 167-bp product; RelB (mouse Chr 7 NM\_009046), forward, CTTTGCCTATGATCCTTC TGC, reverse, GAGTCCAGTGTAGGGGCTCT, 150-bp product; iNOS (mouse Chr 11 NM\_010927), forward, CTAT-CAGGAAGAAATGCAGGAGAT, reverse, GAGCACGCT-GAGTACCTCATT, 145-bp product; ICAM-1 (mouse Chr 9 NM\_010493), forward, GTTAAAAACCAGACCCTGGAA CT, reverse, CGTCTGCAGGTCATCTTAGGAG, 153-bp product; VCAM-1 (mouse Chr 3 NM\_011693), forward, CCGGCATATACGAGTGTGAAT, reverse, ATGGCAGG TATTACCAAGGAAGAT, 151-bp product; GAPDH (mouse Chr 6 NM\_008084), forward, ATGTGTCCGTCGTGGATC TGAC, reverse, AGACAACCTGGTCCTCAGTGTAG 132-bp product. The cycling conditions of RT-PCR were 95°C for 2 min; up to 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec; and a final 4-min extension at 72°C. All products were amplified with *Taq* DNA polymerase (Promega, Madison, WI) by DNA Engine Dyad (Bio-Rad, Hercules, CA). The cDNA samples in each group were diluted 6-fold with DNase-free water and were subjected independently three times to real-time PCR with the same primers of RT-PCR and SYBR Green PCR Master Mix (Applied Biosystems, CA) by using the StepOnePlus Real-Time PCR System (Applied Biosystems, CA). Cycling conditions of real-time PCR were 95°C for 2 min; 40 cycles of 95°C for 30 sec, 60°C for 60 sec. GAPDH was used as a reference gene, and relative quantification analysis was determined via the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

#### Statistical Analysis

Numerical results are presented as means  $\pm$  SD. The significant differences between the data sets were assessed with the Student's *t*-tests. The differences were considered significant at  $P < 0.05$  and/or at  $P < 0.01$ .

## RESULTS

### Response of NF- $\kappa$ B/Rel Family to Acoustic Overstimulation

The expression of the NF- $\kappa$ B/Rel family members, NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, and RelB, was observed in the whole cochlea of the control group at 30 cycles of the reaction in RT-PCR. GAPDH in the control group was also confirmed at 24 cycles and used as an internal control to compare between the control group and the NE 2h+4h group. All members of the NF- $\kappa$ B/Rel family in the NE 2h+4h group were confirmed at 30 cycles and obviously enhanced relative to the control group at the same number of cycles with no difference in GAPDH at 24 cycles (Fig. 1A). We divided the cochleas into the CLW and the RoC to determine whether

TABLE I. Expression of NF- $\kappa$ B/Rel Family Members in CLW and RoC After NE<sup>†</sup>

Material		NF- $\kappa$ B C <sub>T</sub> Ave.	GAPDH C <sub>T</sub> Ave.	Ave. $\Delta$ C <sub>T</sub> (NF- $\kappa$ B C <sub>T</sub> -GAPDH C <sub>T</sub> )	$\Delta\Delta$ C <sub>T</sub> (Ave. $\Delta$ C <sub>T</sub> - Ave. $\Delta$ C <sub>T</sub> CLW control)	Normalized amount relative to RoC control 2 <sup>-<math>\Delta\Delta</math>C<sub>T</sub></sup>
CLW	NF- $\kappa$ B1	28.30	21.32	6.98 $\pm$ 0.11	0.00 $\pm$ 0.11	1.00 (0.93–1.08)
Control	NF- $\kappa$ B2	27.21		5.98 $\pm$ 0.26	0.00 $\pm$ 0.26	1.00 (0.84–1.20)
	RelA	27.25		5.92 $\pm$ 0.18	0.00 $\pm$ 0.18	1.00 (0.88–1.13)
	RelB	26.39		5.07 $\pm$ 0.06	0.00 $\pm$ 0.06	1.00 (0.96–1.04)
CLW	NF- $\kappa$ B1	29.11	22.16	6.95 $\pm$ 0.15	-0.03 $\pm$ 0.15	1.01 (0.92–1.13)
NE2h	NF- $\kappa$ B1	27.85		5.69 $\pm$ 0.20	-0.20 $\pm$ 0.20	1.14 (1.00–1.32)
	RelA	27.63		5.46 $\pm$ 0.21	-0.46 $\pm$ 0.21	1.38 (1.19–1.59)
	RelB	27.06		4.90 $\pm$ 0.31	-0.17 $\pm$ 0.31	1.13 (0.91–1.39)
CLW	NF- $\kappa$ B1	27.84	21.44	6.40 $\pm$ 0.17	-0.58 $\pm$ 0.17	1.49 (1.33–1.68)
NE2h+2h	NF- $\kappa$ B1	26.78		5.34 $\pm$ 0.27	-0.55 $\pm$ 0.27	1.46 (1.21–1.77)
	RelA	26.85		5.41 $\pm$ 0.25	-0.51 $\pm$ 0.25	1.42 (1.20–1.69)
	RelB	26.66		5.22 $\pm$ 0.32	0.15 $\pm$ 0.24	0.90 (0.76–1.06)
CLW	NF- $\kappa$ B1	24.36	19.33	5.03 $\pm$ 0.16	-1.95 $\pm$ 0.16	3.86 (3.46–4.32)
NE2h+4h	NF- $\kappa$ B1	23.60		4.27 $\pm$ 0.15	-1.62 $\pm$ 0.15	3.07 (2.77–3.41)
	RelA	23.92		4.08 $\pm$ 0.25	-1.34 $\pm$ 0.25	2.53 (2.13–3.01)
	RelB	22.50		3.17 $\pm$ 0.32	-1.90 $\pm$ 0.32	3.73 (2.99–4.66)
RoC	NF- $\kappa$ B1	29.74	22.27	7.54 $\pm$ 0.21	0.00 $\pm$ 0.21	1.00 (0.93–1.08)
Control	NF- $\kappa$ B2	28.64		6.37 $\pm$ 0.24	0.00 $\pm$ 0.24	1.00 (0.84–1.20)
	RelA	29.18		6.91 $\pm$ 0.19	0.00 $\pm$ 0.19	1.00 (0.88–1.13)
	RelB	28.34		6.07 $\pm$ 0.26	0.00 $\pm$ 0.26	1.00 (0.96–1.04)
RoC	NF- $\kappa$ B1	29.53	21.96	7.57 $\pm$ 0.21	0.10 $\pm$ 0.21	0.93 (0.93–1.24)
NE2h	NF- $\kappa$ B2	28.09		6.13 $\pm$ 0.37	-0.24 $\pm$ 0.33	1.18 (0.94–1.48)
	RelA	28.62		6.66 $\pm$ 0.32	-0.25 $\pm$ 0.32	1.19 (0.95–1.48)
	RelB	28.17		6.20 $\pm$ 0.42	0.13 $\pm$ 0.42	0.91 (0.68–1.22)
RoC	NF- $\kappa$ B1	29.15	21.90	7.25 $\pm$ 0.20	-0.22 $\pm$ 0.20	1.16 (1.01–1.34)
NE2h+2h	NF- $\kappa$ B2	27.29		5.39 $\pm$ 0.25	-0.98 $\pm$ 0.25	1.97 (1.66–2.35)
	RelA	27.80		5.90 $\pm$ 0.02	-1.01 $\pm$ 0.02	2.01 (1.99–2.31)
	RelB	26.61		4.71 $\pm$ 0.24	-1.36 $\pm$ 0.24	2.57 (2.17–3.03)
RoC	NF- $\kappa$ B1	25.47	19.67	5.80 $\pm$ 0.17	-1.67 $\pm$ 0.17	3.18 (2.83–3.58)
NE2h+4h	NF- $\kappa$ B2	24.79		5.12 $\pm$ 0.19	-1.25 $\pm$ 0.25	2.38 (2.08–2.71)
	RelA	24.98		5.31 $\pm$ 0.20	-1.60 $\pm$ 0.20	3.03 (2.64–3.48)
	RelB	23.90		4.23 $\pm$ 0.28	-1.84 $\pm$ 0.28	3.58 (2.95–4.35)

<sup>†</sup>C<sub>T</sub>, threshold cycle, "Ave.", average ( $\pm$ SD); CLW, cochlear lateral wall, RoC, rest of cochlea. NE, noise exposure.

\* $P < 0.05$ ;

\*\* $P < 0.01$ .

there is any expression difference between the CLW and the RoC. Quantitative real-time PCR analysis indicated that the expression of NF- $\kappa$ B members in the RoC was slightly but statistically increased at 2 hr after NE 2h (NE 2h+2h) (control/NE 2h+2h; CLW:  $P = 0.054$ , RoC: \* $P = 0.025$ ). The significant up-regulation in both the CLW and the RoC was observed at 4 hr after NE 2h (NE 2h+4h) compared with the control group (control/NE 2h+4h; CLW: \*\* $P = 0.003$ , RoC: \*\* $P = 0.002$ ) (Fig. 1B, Table I, Supp. Info. Table I). Therefore, the result shows that the NF- $\kappa$ B/Rel family members were induced in both the CLW and the RoC by acoustic overstimulation (NE: 117 dB/SPL/2 hr), while their initiated time in the CLW was slightly delayed compared with that of the RoC.

### Time Course of Inflammatory Factor Up-regulation in CLW and RoC After Acoustic Overstimulation

To investigate the inflammatory influence of NF- $\kappa$ B signaling on the CLW and the RoC, inducible nitric

oxide synthase (iNOS), intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were selected as the inflammatory factors of the NF- $\kappa$ B-induced target genes. Quantitative real-time PCR analysis showed that in both the CLW and the RoC, the up-regulation of ICAM-1 was first initiated at NE 2h (control/NE 2h; CLW: \* $P = 0.004$ , RoC: \*\* $P = 0.002$ ), and that of iNOS followed at NE 2h+2h (control/NE 2h+2h; CLW: \* $P = 0.008$ , RoC: \*\* $P = 0.0005$ ). In contrast, the induction of VCAM-1 expression was confirmed at NE 2h+2h in the CLW, while lacking significant increase in the RoC (control/NE 2h+2h; CLW: \* $P = 0.006$ , RoC:  $P = 0.027$ ) (Fig. 2A, Table II, Supp. Info. Table II). In the RoC, the up-regulation of ICAM-1 and iNOS reached peak levels at NE2 hr + 2 hr and NE 2h+4h respectively (ICAM-1: 21.26-fold at NE 2h+2h, iNOS: 12.30-fold at NE 2h+4h) and then rapidly declined at NE 2h+6h (Fig. 2A, Table II, Supp. Info. Table II). In contrast, for the target-gene expression patterns in the RoC, all inflammatory factors in the CLW progressively increased until

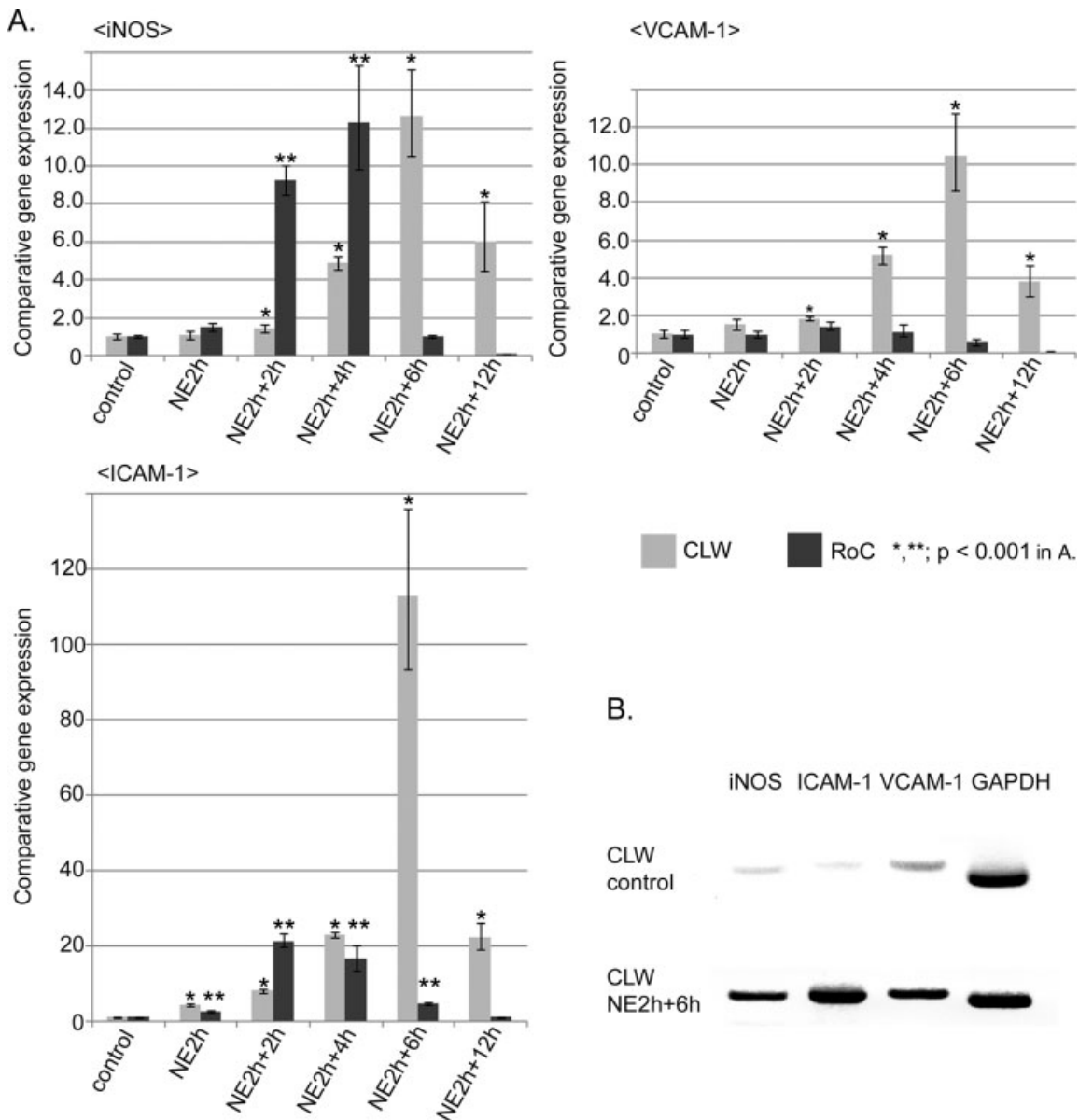


Fig. 2. Time course of inflammatory factors in the CLW after acoustic overstimulation. **A:** Three graphs of the expression of iNOS, ICAM-1, and VCAM-1 in the CLW and the RoC. The time course of the target genes is indicated by the values estimated by the  $2^{-\Delta\Delta C_T}$  method in quantitative RT-PCR analysis. Each value represents a comparison with the control group defined as 1.0. Statistical differences in the data sets between the control group and the NE groups were assessed with Student's *t*-tests and considered significant at  $P < 0.01$  (CLW: \*, RoC: \*\*). **B:** PCR products of iNOS (145 bp), ICAM-1(153 bp), VCAM-1(151 bp), and GAPDH (132 bp) in the CLW by RT-PCR. The intensities of iNOS, ICAM-1, and VCAM-1 were compared at 33 cycles between the CLW control group and the CLW NE 2h+6h group. GAPDH (132 bp) was also performed as a reference gene and compared at 24 cycles. Error bars indicate the SD.

Statistics: (iNOS) control/NE 2h; CLW:  $P = 0.383$ , RoC:  $P = 0.024$ , control/NE 2h+2h; CLW:  $P = 0.008$ , RoC:  $P = 0.001$ , control/NE 2h+4h; CLW:  $P = 0.001$ , RoC:  $P = 0.002$ , control/NE 2h+6h; CLW:  $P = 0.001$ , RoC:  $P = 0.486$ , control/NE 2h+12h; CLW:  $P = 0.002$ , RoC:  $P = 0.001$ , (ICAM-1) control/NE 2h; CLW:  $P = 0.004$ , RoC:  $P = 0.002$ , control/NE 2h+2h; CLW:  $P = 0.0003$ , RoC:  $P = 0.0002$ , control/NE 2h+4h; CLW:  $P = 0.0004$ , RoC:  $P = 0.001$ , control/NE 2h+6h; CLW:  $P = 0.0001$ , RoC:  $P = 0.0004$ , control/NE 2h+12h; CLW:  $P = 0.002$ , RoC:  $P = 0.232$ , (VCAM-1) control/NE 2h; CLW:  $P = 0.092$ , RoC:  $P = 0.335$ , control/NE 2h+2h; CLW:  $P = 0.011$ , RoC:  $P = 0.027$ , control/NE 2h+4h; CLW:  $P = 0.006$ , RoC:  $P = 0.262$ , control/NE 2h+6h; CLW:  $P = 0.0001$ , RoC:  $P = 0.008$ , control/NE 2h+12h; CLW:  $P = 0.009$ , RoC:  $P = 0.004$ .

TABLE II. Expression of iNOS, ICAM-1, and VCAM-1 in CLW and RoC After NE<sup>†</sup>

Material		Target gene C <sub>T</sub> Ave.	GAPDH C <sub>T</sub> Ave.	Ave. ΔC <sub>T</sub> (C <sub>T</sub> -GAPDH C <sub>T</sub> )	ΔΔC <sub>T</sub> (Ave. ΔC <sub>T</sub> - Ave. ΔC <sub>T</sub> CLW (or RoC) control)	Normalized amount relative to CLW (or RoC) control 2 <sup>-ΔΔC<sub>T</sub></sup>
CLW	iNOS	33.31	21.19	12.12 ± 0.21	0.00 ± 0.21	1.00 (0.86-1.16)
Control	ICAM-1	32.42		11.23 ± 0.22	0.00 ± 0.22	1.00 (0.86-1.16)
	VCAM-1	27.22		6.03 ± 0.31	0.00 ± 0.31	1.00 (0.81-1.24)
CLW	iNOS	34.63	22.56	12.07 ± 0.29	-0.05 ± 0.29	1.04 (0.81-1.27)
NE2h	ICAM-1	31.66		9.10 ± 0.11	-2.13 ± 0.11	4.38 (4.06-4.72)*
	VCAM-1	28.00		5.45 ± 0.29	-0.58 ± 0.29	1.49 (1.22-1.83)
CLW	iNOS	35.06	23.46	11.61 ± 0.20	-0.51 ± 0.20	1.42 (4.50-5.24)*
NE2h+2h	ICAM-1	31.70		8.24 ± 0.09	-2.99 ± 0.09	7.94 (7.46-8.46)*
	VCAM-1	28.64		5.18 ± 0.09	-0.85 ± 0.09	1.80 (1.69-1.92)*
CLW	iNOS	34.30	24.28	9.84 ± 0.11	-2.28 ± 0.11	4.86 (4.50-5.24)*
NE2h+4h	ICAM-1	31.25		6.72 ± 0.04	-4.51 ± 0.04	22.78 (22.16-23.43)*
	VCAM-1	28.64		3.66 ± 0.13	-2.37 ± 0.13	5.17 (4.72-5.66)*
CLW	iNOS	32.74	23.01	8.46 ± 0.26	-3.66 ± 0.26	12.64 (10.56-15.14)*
NE2h+6h	ICAM-1	28.69		4.41 ± 0.27	-6.82 ± 0.27	112.99 (93.70-136.24)*
	VCAM-1	26.92		2.64 ± 0.28	-3.39 ± 0.28	10.48 (8.63-12.73)*
CLW	iNOS	29.96	20.43	9.53 ± 0.43	-2.59 ± 0.43	6.02 (4.47-8.11)*
NE2h+12h	ICAM-1	27.18		6.75 ± 0.23	-4.48 ± 0.23	22.32 (19.03-26.17)*
	VCAM-1	24.55		4.12 ± 0.31	-1.91 ± 0.31	3.76 (3.00-4.66)*
Roc	iNOS	31.65	23.01	8.63 ± 0.09	0.00 ± 0.09	1.00 (0.94-1.06)
Control	ICAM-1	33.87		10.86 ± 0.32	0.00 ± 0.32	1.00 (0.80-1.25)
	VCAM-1	28.43		5.42 ± 0.31	0.00 ± 0.31	1.00 (0.81-1.24)
Roc	iNOS	31.62	23.54	8.08 ± 0.21	-0.55 ± 0.21	1.46 (1.27-1.69)
NE2h	ICAM-1	33.06		9.52 ± 0.21	-1.34 ± 0.21	2.53 (2.19-2.93)**
	VCAM-1	29.05		5.50 ± 0.26	0.06 ± 0.26	0.96 (0.80-1.15)
Roc	iNOS	31.16	25.75	5.42 ± 0.12	-3.21 ± 0.12	9.25 (8.51-10.06)**
NE2h+2h	ICAM-1	32.19		6.45 ± 0.12	-4.41 ± 0.12	21.26 (19.56-23.10)**
	VCAM-1	30.65		4.90 ± 0.20	-0.52 ± 0.20	1.43 (1.25-1.65)
Roc	iNOS	30.57	25.67	5.01 ± 0.32	-3.62 ± 0.32	12.30 (9.85-15.35)**
NE2h+4h	ICAM-1	32.49		6.82 ± 0.29	-4.04 ± 0.29	16.45 (13.45-20.11)**
	VCAM-1	30.90		5.23 ± 0.38	-0.19 ± 0.38	1.14 (0.88-1.48)
Roc	iNOS	30.96	22.28	8.63 ± 0.11	0.00 ± 0.11	1.00 (0.93-1.08)
NE2h+6h	ICAM-1	30.96		8.68 ± 0.11	-2.18 ± 0.11	4.53 (4.20-4.89)**
	VCAM-1	28.34		6.06 ± 0.15	0.64 ± 0.15	0.64 (0.37-0.71)
Roc	iNOS	33.66	20.85	12.81 ± 0.36	4.18 ± 0.36	0.06 (0.04-0.07)
NE2h+12h	ICAM-1	31.84		10.99 ± 0.32	-0.13 ± 0.32	0.91 (0.73-1.14)
	VCAM-1	28.42		7.57 ± 0.34	2.15 ± 0.31	0.23 (0.18-0.28)

<sup>†</sup>C<sub>T</sub>, Threshold cycle; Ave, average (±SD); CLW, cochlear lateral wall; RoC, rest of cochlea; NE, Noise exposure.

\*,\*\*P < 0.01.

NE 2h+6h, and slowly decreased at NE 2h+12h (Fig. 2A). In particular, the adhesion molecules were remarkably up-regulated in the CLW. The peak levels of iNOS, ICAM-1, and VCAM-1 reached 12.64-fold, 112.99-fold, and 10.48-fold, respectively, at NE 2h+6h (Fig. 2A, Table II, Supp. Info. Table II). The RT-PCR result also indicated the faint expression of iNOS, ICAM-1, and VCAM-1 in the CLW control group at 33 cycles of the reaction and the substantial increases of all factors in the CLW NE 2h+6h group at the same cycles (Fig. 2B). In contrast, there was no difference in GAPDH at 24 cycles between the two groups (Fig. 2B). Thus, these data indicated that acoustic overstimulation (NE: 117 dB/SPL/2 hr) induced different NF-κB target gene expression patterns in the values and the varieties between the CLW and the RoC.

### Expression of Adhesion Molecules in CLW by Acoustic Overstimulation

To determine the changes in the adhesion molecules (ICAM-1 and VCAM-1) at the protein level, the CLW tissues in the NE 2h+14h group were prepared for immunohistochemistry and compared with that of the control group. The analysis time point of NE 2h+14h was selected as a sufficient interval to produce the proteins following the peak of target gene expression. Figure 3 shows that the adhesion molecules were slightly expressed in the stria vascularis (SV) under the normal condition. The result was compatible with their expression in the control group by RT-PCR (Fig. 2B). Although ICAM-1 and VCAM-1 were widely expressed in the CLW of the control group, their expression was

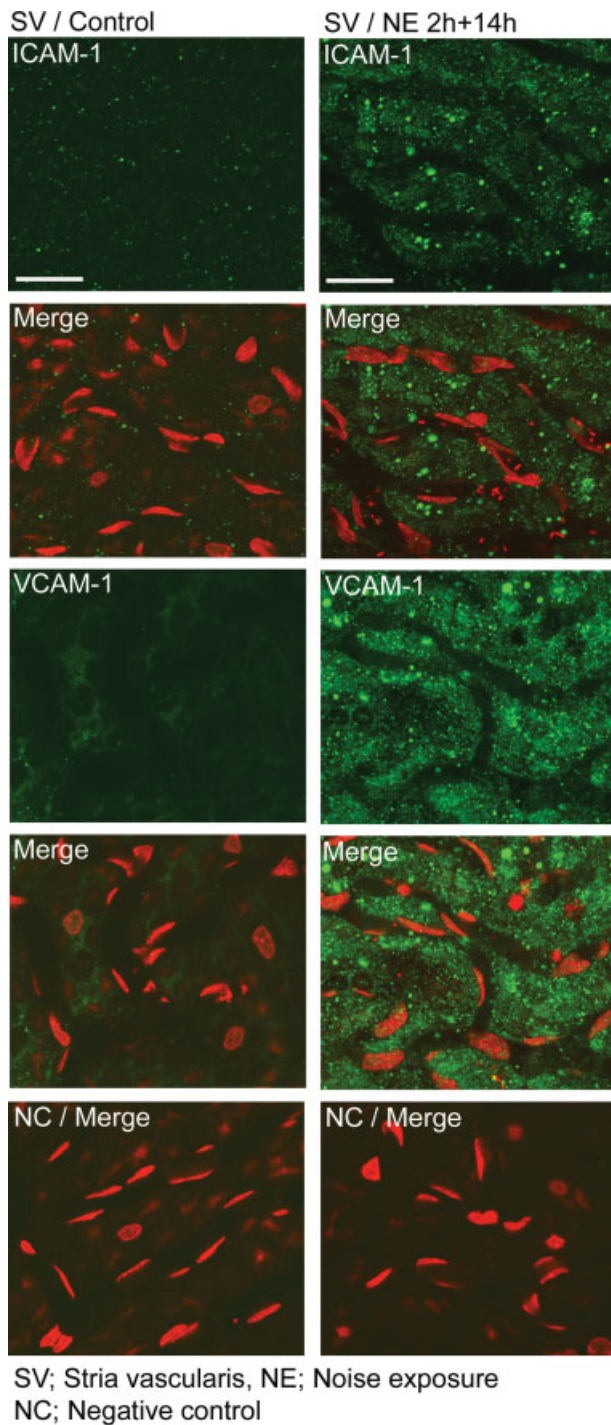


Fig. 3. Expression of adhesion molecules in the SV. Panels are fluorescent confocal images from whole mount preparations of the CLW. The tissues were immunolabeled with ICAM-1 or VCAM-1 (green) and the nuclei were stained with PI (red). All images were observed at the SV level. Merged images were compared between the control group and the NE 2h+14h group. Faint expression of ICAM-1 and VCAM-1 was observed under normal condition in comparison with negative controls. Scale bars = 20  $\mu$ m.

remarkably up-regulated by acoustic overstimulation. In particular, the labels of both ICAM-1 and VCAM-1 were most prominent at the marginal cell membranes and the processes of intermediate cells associated with capillaries in the SV (Fig. 3). The expression of VCAM-1 is more intense than that of ICAM-1 in the SV and their expressions coincided with each other. The negative controls (NC) in both the control group and the NE 2h+14h group showed no antibody labeling.

## DISCUSSION

In the present study, we have shown that acoustic overstimulation affects the transcription level of the NF- $\kappa$ B/Rel family members (NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, and RelB) in the murine inner ear and the inflammatory factors (iNOS, ICAM-1, and VCAM-1) triggered by the NF- $\kappa$ B signaling are up-regulated in the CLW and the RoC. In the time-course study, the up-regulation of the inflammatory factors presented as bimodal peaks between the CLW and the RoC that originated from the slightly delayed response of NF- $\kappa$ B to acoustic overstimulation in the CLW. The early initiation of ICAM-1 expression, apparently preceding the transcriptional change of NF- $\kappa$ B, implies its high sensitivity to the NF- $\kappa$ B signaling, which derives from activating processes of NF- $\kappa$ B at the protein level (i.e., phosphorylation and cleavage of NF- $\kappa$ B/inhibitory protein complexes in the cytoplasm). However, the later and robust response of ICAM-1 and VCAM-1 at the transcription level correlated with their large expression in the CLW at the protein level. Specifically, ICAM-1 and VCAM-1 were overexpressed in the SV, which is the most active ion transporting region in the CLW and plays a key role in regulating ionic balance and producing endocochlear potential (EP) in the endolymph (Wangemann, 2002; Nin et al., 2008).

The NF- $\kappa$ B/Rel family members, which are constitutively produced through mRNA translations, are degraded through proteolytic processes at the protein level and generally exist as inactivated forms with inhibitory proteins in the cytoplasm. In response to diverse stimuli, the NF- $\kappa$ B signal-transduction pathways are activated by phosphorylation and ubiquitin-dependent cleavage of NF- $\kappa$ B/inhibitory protein complexes (Ghosh et al., 1998; Siebenlist et al., 2005; Ghosh and Karin, 2002). In the light of evidence, the transcriptional response of NF- $\kappa$ B to acoustic overstimulation might be inadequate to implicate the whole process of the activated NF- $\kappa$ B signal-transduction pathways. However, other evidence that NF- $\kappa$ B signals are constantly activated in prolonged and excessive stimulated cells (Hart et al., 1998; Karin et al., 2002) supports our view that the progressive production of NF- $\kappa$ B/Rel family at the transcription level is potentially associated with its activation process. Therefore, in the present study, the up-regulation of the NF- $\kappa$ B/Rel family at the transcription level implicates an activation of the NF- $\kappa$ B signal-transduction pathways. Besides the elevations of NF- $\kappa$ B1 and RelA, known as predominant forms in the canonical

pathway, the rise of NF- $\kappa$ B2 and RelB in the non-canonical pathway also implies that both NF- $\kappa$ B signal-transduction pathways are potentially activated in response to acoustic overstimulation.

In the inner ear, acoustic overstimulation is well-known to cause the apoptotic degeneration of cochlear hair cells and spiral ganglion cells (Lang et al., 2006; Nagashima et al., 2007) as well as the morphological and physiological alteration in the CLW (Hirose and Liberman, 2003). In the SV, which is composed of three different cell types (marginal cells, intermediate cells, and basal cells), and with well-developed capillaries, acoustic overstimulation produces dramatic cellular damage such as swollen marginal cells, shrunken intermediate cells, and enlarged intrastrial spaces within 24 hr (Hirose and Liberman, 2003). This damage leads to the severe physiological changes of EP and ionic balance in the endolymph for several days (Konishi et al., 1979; Hirose and Liberman, 2003). It is noteworthy that the prominent expression of ICAM-1 and VCAM-1 in the SV were consistent with the region, which is preferentially vulnerable to acoustic stimulation (Hirose and Liberman, 2003). These findings support the view that there must be a link between the inflammatory factors triggered by NF- $\kappa$ B signaling and the histopathological changes in the SV.

A number of past studies have already shown that iNOS-induced ROS causes cellular and DNA damage in the organs by compromising the structural and functional integrity of the cell membrane and promoting mutagenic deamination of DNA bases (Moncada et al., 1991; Collins et al., 1995; Cuzzocrea et al., 2001; Shi and Nuttall, 2003). Besides, adhesion molecules affect hemodynamics by leukocyte-endothelial cell interactions such as leukocyte rolling, adhesion, and transmigration in the inflamed tissues, which ultimately lead to severe energy depletion of the cells and necrotic-type cell death (Miwa et al., 1997; Ridker et al., 1998; Shi and Nuttall, 2007; Sumagin and Sarelius, 2007). More importantly, a recent study provides evidence that ICAM-1 also plays an essential role in regulating microvascular permeability, demonstrating that the overexpression of ICAM-1 significantly increases the microvascular permeability in wild type mice, whereas in ICAM-1 deficient animals, the permeable change is abolished (Sumagin et al., 2008). This evidence leads us to speculate that the cytotoxic potential of iNOS and the hemodynamic change by ICAM-1 and VCAM-1 influence cellular integrity and energy metabolism in the SV. Moreover, the microvascular hyperpermeability by overproduction of ICAM-1 may contribute to the enlarged intrastrial spaces, where extensive ramified membranes of marginal cells interdigitate with processes of intermediate cells and capillaries in order to form the complex networks for ion transportation and EP production. In accordance with the NF- $\kappa$ B/Rel family as an essential transcription factor for innate and adaptive immunity, NF- $\kappa$ B signaling must have a vital role in cochlear hair cell maintenance and normal hearing (Nagy et al., 2005; Lang et al., 2006). Its

excessive influence on immune responses, however, frequently leads to self-injurious phenomena such as allergic diseases (Hart et al., 1998) and secondary inflammatory injury by ischemia/reperfusion (Cuzzocrea et al., 2001; Valen et al., 2001; Conti et al., 2007). On the basis of the evidence, our study suggests that the excess response of NF- $\kappa$ B signals to acoustic overstimulation causes marked expression of the inflammatory factors in the inner ear and the hemodynamics and the cellular integrity in the SV are potentially impacted by the adhesion molecules (ICAM-1 and VCAM-1) as well as iNOS. Thus, the regulation of NF- $\kappa$ B signaling might be meaningful as a future therapeutic target to acoustic trauma and further studies are needed to elucidate the control of NF- $\kappa$ B signaling and the regulation of the specific target gene expressions in the inner ear.

In conclusion, this study provides new information that the NF- $\kappa$ B/Rel family members NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, and RelB, as the predominant forms in two signal-transduction (canonical and noncanonical) pathways, are induced in the murine inner ear by acoustic overstimulation and the induced inflammatory factors (iNOS, ICAM-1, and VCAM-1) produced in the CLW are probably associated with the subsequent histopathological changes in the SV.

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