Involvement of NLRP3-inflammasome pathway in noise-induced hearing loss

Abstract

The inflammasome is a multiprotein oligomer in the cell cytoplasm and is part of the innate immune system. It plays a crucial role in the pathological process of noise-induced hearing loss (NIHL). However, the mechanisms of NLR family pyrin domain containing 3 (NLRP3) inflammasome activation in NIHL have not been clearly demonstrated. In this study, miniature pigs were exposed to white noise at 120 dB(A) and auditory brainstem response measurements were used to measure their hearing function. Immunofluorescence staining, confocal laser scanning microscopy, western blot assay, and quantitative reverse transcription-polymerase chain reaction were used to analyze inflammasome-related protein distribution and expression. NLRP3, interleukin-1β, interleukin-18, and cleaved-caspase-1 were highly expressed in the cochlea after 120 dB(A) white noise exposure. Our findings suggest that NLRP3-inflammasomes in the cochlea may be activated after acoustic trauma, which may be an important mechanism of noise-induced hearing loss.

Key Words: acoustic injury; inflammasome; interleukin-1β; miniature pig; NLR family pyrin domain containing 3 (NLRP3); noise-induced hearing loss; sensorineural hearing loss

Introduction

Sensorineural hearing loss (SNHL) is an increasingly common problem in the world, and noise-induced hearing loss (NIHL) is the second most common cause of nonhereditary hearing impairment (Kurabi et al., 2017; Zheng and Zuo, 2017). It has been suggested that more than 600 million people worldwide live with NIHL (Le et al., 2017).

Studies have shown that acoustic injury of the auditory system can be caused by multiple factors, including mechanical damage of hair cells, microcirculation vascular damage, and metabolic disorders that lead to hair cell death (Cai et al., 2014; Zheng and Zuo, 2017; Wang and Puel, 2018). Several recent studies have reported that the immunological and inflammatory responses to acoustic overstimulation in the cochlea play a crucial role in the development of NIHL (Cai et al., 2014; Vethanayagam et al., 2016; Yang et al., 2016; Frye et al., 2019). Previously, we reported an increase of reactive oxygen species (ROS) and nuclear factor kappa-B (NF-κB) nuclear translocation during the acute hearing loss stage in miniature pig inner ear hair cells following acoustic trauma (Sai et al., 2020).

The inflammasome is a multiprotein oligomer and part of the innate immune system. Its primary components include pattern recognition receptors (PRRs), apoptosis-associated speck-like protein containing CARD (ASC), and procaspase-1 (Martinon et al., 2002). Researchers have identified a number of PRRs involved in inflammasomes, including NOD-like receptors (NLRs) family pyrin domain, such as NLRP1, NLRP3, NLRP6, NLRP7, and NLRP12, CARD domain containing 4 (NLRC4), absence in melanoma 2 (AIMP2), IFI16, and RIG-I (Schröder and Tschopp, 2010). Inflammasomes form in response to a wide range of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs). Through the self-cleavage of procaspase-1, the inflammasome activates caspase-1, which causes the maturation of interleukins 1β and 18 (IL-1β and IL-18) (Malik and Kanneganti, 2017). IL-1β and IL-18 are released extracellularly for participation in inflammation, injury, and other processes (Gross et al., 2011). Several recent investigations have shown that SNHL is linked to inflammasome activation (Martinon et al., 2009; Kuehmerle-Deschner et al., 2015; Shi et al., 2015, 2017; Zhuang et al., 2018). Nevertheless, the data indicating that inflammasomes play a role in NIHL development are limited. The aim of this study was to explore the involvement of the NLRP3-inflammasome pathway in the regulation of noise-induced cochlear injury.

Materials and Methods

Animals

Twenty healthy miniature pigs (2–3 months old, weighing 5–6 kg) were...
purchased from Zhuzhou Kangning Miniature Pig Cultivation Company (Hebei, China, license No. SYXX (Ji) 018-003). To avoid the effects of individual differences on the study results, only male pigs were used in this study, and a baseline hearing test using auditory brainstem response (ABR)-click was performed on all animals. There were ten pigs in the normal control group and ten pigs in the noise exposure group. Following the baseline hearing test, animals were randomly allocated to either the noise exposure or the control group (using a random number table). All cochleae were subjected to a 12-hour light-dark cycle and had access to food and water ad libitum. Procedures involving the use and care of animals were approved by the Institutional Animal Care and Use Committee of Chinese PLA General Hospital (approval No. 201709) and were conducted in strict accordance with international laws and National Institutes of Health (NIH) policies, including the Guide for the Care and Use of Laboratory Animals (8th ed, 2011). This study was reported in accordance with the ARRIVE 2.0 guidelines for Animal Research: Reporting of In Vivo Experiments (Percie d’Seu, 2020).

Noise exposure

The animals in the noise exposure group were placed in a wire mesh cage and subjected to 3 hours of white noise at 120 dBA for 2 consecutive days. The control group did not receive noise treatment. The noise signal was generated using a signal processor (RZ6, Tucker Davis Technologies [TDT], Alachua, FL, USA). The signal was routed through an attenuator (PAS, TDT) and an amplifier (Crown, XL5402, Pittsburgh, PA, USA) to a loudspeaker (Zhenmei Co., Ltd., Jiangsu, China), which was placed 20 cm above the animal’s head. This noise exposure regimen resulted in a permanent loss of cochlear sensitivity as described in our previous research (Wu et al., 2017; Sai et al., 2020).

Auditory brainstem response testing

To assess hearing function, we measured ABR before and one day after noise exposure. A 5-cm animal was put in a sound-proof chamber after being sedated with an intramuscular injection of xylazine hydrochloride (0.1 mL/kg; Shengda Reagent Co., Ltd., Jilin, China) and under anesthesia with 3% pentobarbital sodium (1 mL/kg, intraperitoneal injection; Sigma, St. Louis, MO, USA). The same method of anesthesia was used in all experiments in this study. A warming blanket was used to keep the body temperature at 38°C. Subdermal stainless-steel needle electrodes were inserted at the vertex (noninverting input) and behind the stimulated and non-stimulated ears (inverting input and ground, respectively). An open-field sound delivery device positioned 1 cm from the animal’s tested ear was used to stimulate each ear independently. Clicks and tone bursts at 2, 4, 8, 16, and 24 kHz were used to elicit ABRs. Whole (SigGien, TDT) with a multifunctional processor (MEDUSA42, TDT). The procedures and parameter settings were similar to those used in previous reports (Hu et al., 2012; Wu et al., 2017). The ABR threshold was established as the weakest stimulus intensity that dependably generated a detectable signal.

Cochlear tissue collection

After the ABR tests, cochleae were removed for gene expression and pathological examination of each group. The animals were heavily anesthetized with pentobarbital sodium and then decapitated. As previously reported, the cochleae were rapidly detached from the skull (Chen et al., 2016). The cochleae were perfused with an RNA stabilization reagent (RNeasy Miniprep Kit, Qiagen, Valencia, CA, USA) to examine the transcriptional expression patterns of the NLRP3-inflammasome and related genes of inflammatory factors. The cochleae were promptly frozen in liquid nitrogen for 10 minutes after being washed with 0.01 M phosphate-buffered saline (PBS) to prepare the brain tissues for RNA extraction. The cochleae were preserved in 4% paraformaldehyde overnight at 4°C for immunohistochemical and pathological investigations. The organ of Corti and stria vascularis were harvested after the dissection of the cochlea in PBS for immunofluorescent staining.

Immunofluorescence staining

We used immunofluorescence staining to observe the immunoreactivity of caspase-1 and IL-1β in the cochlea. Tissue processing and immunofluorescence of the cochleae were performed as previously reported (Wu et al., 2017; Sai et al., 2020). After the organ of Corti and stria vascularis were separated, the tissues were permeabilized for 15 minutes in PBS with 0.25% Triton X-100, blocked for 30 minutes in PBS with 5% goat serum, and then incubated overnight at 4°C with the appropriate primary antibody (caspase-1: rabbit, 1:100, Abcam, Cambridge, MA, USA, ab-104279; IL-1β: rabbit, 1:100, Abcam, ab-104279; NF-kB p65: rabbit, 1:100, Abcam, Cat# ab7737, RRID: AB_306031; anti-NF-κB p65 (H+L), horseradish peroxidase-conjugated, 1:1000, TDY Biotech Co., Ltd., Beijing, China; Cat# 50001, goat-anti-rabbit IgG (H+L), horseradish peroxidase-conjugated, 1:1000, TDY Biotech Co., Ltd., Cat# 50004). Antibodies were detected using the chemiluminescence substrate (32109, ECL Plus; Amersham Bioscics, Piscataway, NJ, USA) after each antibody preparation was diluted in 5% skim milk (WBKLS0514, USA). The immunoactive protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA; Schneider et al., 2012). Protein expression was normalized to β-actin.

Quantitative reverse transcription-polymerase chain reaction

Total RNA from each pig’s cochlea was separately extracted with RNeasy (Qiagen, Venlo, Netherlands). Each group of four pigs (eight cochleae) was analyzed for NLRP3, Caspase-1, IL-1β, IL-6, NF-kB p65, TNF-α and GAPDH mRNA with quantitative reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA (cDNA) was synthesized from 5 μg total RNA using the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), PCR amplification of the cDNA and quantification was performed using SYBR (Thermo Fisher Scientific). The PCR conditions were as follows: holding the fluorescent signal for 1 cycle at 95°C for 20 seconds, followed by 40 denaturation cycles at 95°C for 3 seconds and annealing at 59°C for 30 seconds. GAPDH as an internal standard was arbitrarily assigned a value of 1.0. The 2-ΔΔΔCT method was used to analyze the relative product levels of quantitative RT-PCR gene expression data. The expression of each gene was normalized to the expression of the endogenous control β-actin (Hu et al., 2012). The expression of each gene in each group was determined as the mean fold change relative to the normal control group, which was set as 1.0. The sequence-specific primers for quantitative RT-PCR were shown in Table 1.

Table 1 | The sequences of primers

| Gene       | Primer sequence (5’→3’) | Product size (bp) |
|------------|-------------------------|-------------------|
| NLRP3      | F: GAC TCT AGG CAA GAT CAC GA  | 123               |
|            | R: TCT GAT GCC CAG TCC AAC AT |                   |
| Caspase-1  | F: CCA GTA ACT CTC AGG TT  | 149               |
|            | R: GGA GAC GCA GCA TTA ACT GG |                   |
| NF-kB p65  | F: TCA TGC ACC TCA CCC ATC GAC | 159               |
|            | R: CAC ACA TGC AAG ATG TAT GG |                   |
| IL-8       | F: CAC ATG TCA AGG CTC TG  | 171               |
|            | R: GGG TGC GGC TAT CCT TTG TCG |               |
| IL-1β      | F: CGC TGA ACA GCT AAT GTC  | 159               |
|            | R: AAG ATG GTC CTA GGT TCT AAG |               |
| TNF-α      | F: GGA TGC AAC CTC TCT GCT  | 98                |
|            | R: CTC CCC AGG ATG AGG ATT |                   |
| GAPDH      | F: TGA ACT GCT ACC TCA CTT G  | 65                |
|            | R: TCA GCT GGA TCG CCA TCT TT |                 |
| Actin      | F: TGA GCT GAC CAT CAC ATC  | 105               |
|            | R: GAT GTC GAG AAG CCA GAT |                   |

F: Forward; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; NLRP3: NLR family pyrin domain containing 3; NF-kB: nuclear factor kappa-B; R: reverse; TNF-α: tumor necrosis factor-α.
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Figure 1A

100

No noise-induced activation of NLRP3-inflammasomes in pig cochleae.

Figure 1B

P

Noise-induced cochlear trauma and hearing function changes.

Figure 2A–C

When compared with the control group, the NE animals had a statistically significant difference (∗P < 0.05; Figure 1C–E). In addition, the noise exposure group had a significantly lower number of SGCs compared with the control group (P < 0.05; Figure 1E).

Figure 1C

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications (Hu et al., 2012; Wu et al., 2017). The mean ± standard deviation was used to express the data. Protein expression, average ABR thresholds, and immunoreactivity for the control and noise exposure groups were compared with Student’s t-test using SPSS version 19.0 (IBM, Armonk, NY, USA). P < 0.05 was considered statistically significant.

Results

Noise exposure causes loss in cochlear sensitivity

The average hearing threshold of the normal control group was 21.5 ± 4.7 dB sound pressure level (SPL), and was 70.5 ± 5.5 dB SPL in the noise exposure group (Figure 1A). The threshold shifts were most significant at 4 kHz, and high-frequency hearing loss was more severe than low-frequency hearing loss (Figure 1B), which is consistent with acute NIHL-associated human hearing function (Le et al., 2017).

To determine the effect of acoustic trauma on sensory cell loss, specifically hair cells (HCs) and spiral ganglion cells (SGCs), we quantified the number of missing cells in the normal control and noise-exposed (NE) cochleae (Figure 1C–E). When compared with the control group, the NE animals had an 85.9% increase in the total number of missing outer hair cells (OHCs), with a statistically significant difference (P < 0.01; Figure 1C and D). The increase in sensory cell damage caused by excessive noise occurred mostly in the middle and basal portions of the cochlear sensory epithelium (40–100% distance from the apex), which was consistent with the hearing function changes (Figure 1F). In addition, the noise exposure group had a significantly lower number of SGCs compared with the control group (P < 0.05; Figure 1E).

Figure 2 | Noise-induced cochlear trauma and hearing function changes.

(A, B) Auditory brainstem response (ABR) click thresholds (A) and ABR tone-burst thresholds (B) in the noise exposure (NE) and normal control (Ctrl) groups. (C) The distance from the apex of the cochlea to the hook process is 0–100% (apex = 0%, base = 100%). Cochleogram distribution of the missing outer hair cells between the two groups in the cochlea. (D) The number of missing outer hair cells (OHCs) per cochlea. (E) Substantial reduction in the number of spiral ganglion cells (SGCs) caused by noise exposure. ∗P < 0.05, **P < 0.01, vs. control group (Student’s t-test), n = 10 in each group in A and B, n = 5 in each group in C–E, where n denotes the number of cochleae. All experiments were repeated six times.

Figure 3 | IL-1β and IL-18 expression levels increased after cochlear acoustic trauma.

One day after NE, western blot analysis (A) showed that both IL-1β and IL-18 were increased in the noise exposure (NE) group compared with the normal control group (Ctrl). Quantification using Image J software (B) of blots in A. In each group, n = 8, where n denotes the number of cochleae. (C) Quantitative reverse transcription-polymerase chain reaction showed increased mRNA of NLRP3 and caspase-1 in the NE group compared with the Ctrl group. In each group, n = 8, where n denotes the number of cochleae. ∗P < 0.05; **P < 0.01 (Student’s t-test). Immunofluorescence showed that caspase-1 was increased in SGN cells (yellow arrows) (D) and hair cells (E) in the NE group (white arrow) compared with the Ctrl group. (F) Mean fluorescence intensity of caspase-1 in the SGNs and hair cells. All experiments were repeated six times. IHC: inner hair cell; OHC: outer hair cell; OHC1, 2, 3: the outer hair cells in columns 1–3; SGN: spiral ganglion.
The NLRP3-inflammasome and related inflammatory factors are involved in various cellular processes linked to SNHL (Shi et al., 2015; Nakanishi et al., 2017; Shi et al., 2017; Zhuang et al., 2018; Feng et al., 2020). Here, we provide evidence that the NLRP3-inflammasome pathway is involved in the regulation of noise-induced cochlear injury. Furthermore, our findings suggest that the downstream inflammatory components of the NLRP3-inflammasome could be investigated as potential therapeutic targets in the treatment of noise-induced cochlear dysfunction and sensory cell destruction.

In this study, miniature pigs were exposed to 120 dB(A) white noise to establish the SNHL model. With the exception of other primates, pigs share the closest evolutionary kinship to humans. In particular, the pig inner ear anatomy is similar to the structure of the human inner ear (Yang, 2016; Zhong et al., 2018). After noise stimulation, the most severe hearing loss in pigs occurred at 4 kHz, which is consistent with the affected frequencies in humans (Chen et al., 2016). Chen et al. established a model of explosive deafness using 145 dB SPL impulse noise exposure (Chen et al., 2016). In the study, the average hearing threshold raised by more than 70 dB SPL after 50 exposures to impulse noise, which is substantially greater than the 49 dB SPL difference found between the white noise exposure group and control group in our investigation. This discrepancy suggests that impulse noise may cause more serious inner ear damage. Furthermore, although the structure of the organ of Corti was unaltered in a previous study, the inner and outer hair cells were partially damaged (Chen, 2014). However, there was no evident loss of IHCs in the present study, and only a partial loss of OHCs and SGCs were observed in the noise exposure group cochleae. This suggests that the damage to the cochlea from the impulse noise injury was primarily mechanical, whereas the predominant damage to the cochlea from the 120dB(A) white noise was metabolic.

The inner ear was once thought to be an "immune privileged" organ because of the presence of the blood-labyrinth barrier. However, the inflammatory/immune response has been considered as a key mechanism of NIHL and the primary response to the noise-stimulated cochlea (Vethanayagam et al., 2016; Yang et al., 2016; Hu et al., 2018; Sai et al., 2020). In the present study, acoustic damage activated the NLRP3-inflammasome in pig cochleae. IL-1β and IL-18 mRNA and protein levels were also increased in the HCs, SGCs, and SVs of the noise exposure group compared with those in the control group. The expression of NLRP3 in cells was very low in the control animals. NLRP3-inflammasome activation is thought to require two signals. The first signal, the pre-stimulation signal, interacts with Toll-like receptors (TLRs) and activates NF-κB pathways (Vethanayagam et al., 2016). The second signal, including PAMPs and DAMPs, is recognized by NLRP3, then ASG are recruited to induce procaspase-1 self-cleavage, and finally, caspase-1 triggers downstream IL-1β and IL-18 activation and release, or induces cell pyroptosis (Bauerfeind et al., 2009; Schroder and Tschopp, 2010; Zhou et al., 2011). We believe that the secondary inflammasome signals produced by acute injury are: 1) extracellular ATP stimulation-induced opening of potassium channels, resulting in intracellular K" gene silencing and Ca" influx (Ayna et al., 2012); 2) lysosomal enzyme release (Eisenbarth and Flavell, 2009; Duwel et al., 2010); and 3) excessive ROS accumulation in cells.

In conjunction with our prior research (Sai et al., 2020), we speculate that excessive ROS buildup is a key element in NLRP3-inflammasome activation. Furthermore, ROS inhibitors or scavengers have been shown to prevent NLRP3-inflammasome activation (Dostert et al., 2008), it has been reported that the proinflammatory cytokine IL-1β increased the number of leukocyte-injured neurons and caused NO, TNF-α, and IL-6 to be secreted from microglia, resulting in neurotoxicity (Frye et al., 2019; Wang et al., 2019). Thirdly, the NLRP3-inflammasome and its downstream factors, NF-κB and IL-1β, can further promote the secretion of inflammatory factors such as TNF-α and IL-6. In our study, we also found that NF-κB, TNF-α, and IL-6 were increased in the noise exposure group. TNF-α and IL-6 combine with TLRs to activate the NF-κB signaling pathway to induce apoptosis and necrosis. As a first signal, the latter can activate the inflammasome. The auditory nervous system is harmed by downstream inflammation and ROS, resulting in NIHL. The pathway mentioned above may form in the cochlea and exacerbate the initial inflammatory response (Figure 5).

Under normal conditions, the NLRP3-inflammasome plays a vital role in defending the body from external bacterial and viral infection, and maintaining homeostasis as part of the innate immune response (Martinson et al., 2009). However, if its activation becomes uncontrollable, an excessive inflammatory response occurs, such as NLRP3 gene mutation, which can lead to Muckle-Wells syndrome (MWS), family Mediterranean fever, and other diseases. Previous studies of MWS reported that IL-1β inhibitors improved or stabilized the hearing function of the majority of patients through developing progressive SNHL (Kuemmerle-Deschner et al., 2015; Tran, 2017; Marchica et al., 2018). Furthermore, it has been confirmed that the auditory threshold elevation began primarily at higher frequencies (Koitschev et al., 2012), suggesting that the initial location of pathologic trauma to the inner ear in MWS patients may be in the basal turn of the cochlea, which is consistent with hearing function changes in NIHL (Le et al., 2017). In conclusion, we believe that noise-induced pathogenesis of hearing impairment, like in MWS, appears to be related to excessive activation of the NLRP3-inflammasome, excessive production of IL-1β, and downstream inflammation. Thus, we believe that NLRP3-inflammasome inhibitors or interleukin-1 inhibitors have high potential as therapeutic targets for NIHL prophylaxis.

**Limitations**
This study did not investigate whether attenuation of inflammasome activity or anti-inflammatory therapy would lead to preservation of the ABR threshold or OHC and SGC numbers within this model. We will investigate whether anti-inflammatory or NLRP3-inflammasome-targeted drugs lead to protection of hearing function, OHCs and SGCs in future research.

**Conclusion**
In this study, a pig model of noise-induced hearing loss was established using 120 dB(A) white noise. White noise mainly caused high-frequency hearing loss at frequencies above 4 kHz. Noise exposure activated the NLRP3-inflammasome in the cochlea, and increased IL-1β and IL-18 production. In addition, the expressions of NF-κB and its downstream inflammatory factors IL-6 and TNF-α were upregulated. The findings suggest that the NLRP3-
inflammasome and its downstream inflammatory factors promote the secretion of inflammatory factors such as TNF-α and IL-6, which activate the NF-κB signaling pathway by combining with TLRs to induce apoptosis and necrosis. The NF-κB signaling pathway could be the first signal to activate the inflammasome and provoke the above-mentioned circulation in the cochlea, which could aggravate the initial inflammatory response and neurotoxicity, and aggravated the cochlear damage. Therefore, inflammasome activation is likely an important molecular mechanism involved in the acoustic damage of the cochlea in miniature pigs, which suggests potential therapeutic targets for NIHL.

Author contributions: NS, WJH, and WWG designed the study; NS, YYY, LM, DL, and QJQ performed the experiments; NS, WJH, and WWG analyzed the data, and wrote the manuscript. All authors approved the final version of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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