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ORGAN TOXICITY AND MECHANISMS

Apelin-13 protects against cisplatin-induced ototoxicity by inhibiting apoptosis and regulating STAT1 and STAT3

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Abstract

The ototoxic side effect of cisplatin is a main cause of sensorineural hearing loss. This side effect limits the clinical application of cisplatin and affects patients' quality of life. This study was designed to investigate the effect of apelin-13 on cisplatin-induced C57BL/6 mice hearing loss model and explore the potential underlying molecular mechanisms. Mice were intraperitoneally injected with 100 μg/kg apelin-13 2 h before 3 mg/kg cisplatin injection for 7 consecutive days. Cochlear explants cultured in vitro were pretreated with 10 nM apelin-13 2 h prior to 30 μM cisplatin treatment for another 24 h. Hearing test and morphology results showed that apelin-13 attenuated cisplatin-induced mice hearing loss and protected cochlear hair cells and spiral ganglion neurons from damage. In vivo and in vitro experimental results showed that apelin-3 reduced cisplatin-induced apoptosis of hair cells and spiral ganglion neurons. In addition, apelin-3 preserved mitochondrial membrane potential and inhibited ROS production in cultured cochlear explants. Mechanistic studies showed that apelin-3 decreased cisplatin-induced cleaved caspase 3 expression but increased Bcl-2; inhibited the expression of pro-inflammatory factors TNF-a and IL-6; and increased STAT1 phosphorylation but decreased STAT3 phosphorylation. In conclusion, our results indicate that apelin-13 could be a potential otoprotective agent to prevent cisplatin-induced ototoxicity by inhibiting apoptosis, ROS production, TNF-α and IL-6 expression, and regulating phosphorylation of STAT1 and STAT3 transcription factors.

Keywords Apelin-13 · Ototoxicity · Hair cells · Spiral ganglion neurons · Apoptosis · STAT1/3

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Abbreviations

STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
HCs	Hair cells
SGNs	Spiral ganglion neurons
ABR	Auditory brainstem response
HE	Hematoxylin and eosin staining
TUNEL	TdT-mediated dUTP nick-end labeling
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor α
IL-6	Interleukin-6
P-STAT1	Phosphorylated signal transducer and activator of transcription 1
P-STAT3	Phosphorylated signal transducer and activator of transcription 3
ELISA	Enzyme-linked immunosorbent assay
MAPK/Akt	Mitogen-activated protein kinase/ protein kinase B

Introduction

The ototoxic side effect of cisplatin is a main cause of sensorineural hearing loss that manifests as an irreversible progressive bilateral progression, adversely affecting the personal and social life of patients, especially pediatric patients (Paken et al. 2019). Although it is well established that the hair cells (HCs) in the organ of Corti are the main cochlea target of cisplatin, it has been suggested that cisplatin can simultaneously damage SGN in the cochlea, and the precise molecular mechanism underlying cisplatin-induced hearing loss remains to be elucidated (Gentilin et al. 2019; Rybak et al. 2019). In addition to the well-known cisplatin-induced apoptosis and oxidative stress in cochleae, recent studies in auditory cells and animal models of hearing loss suggest that damage to HCs and SGNs involves cisplatin-induced inflammation (Ramkumar et al. 2021). Several studies show that cisplatin increases the expression of the pro-inflammatory cytokines TNF-α and IL-6 in the inner ear. Neutralizing TNF-α before cisplatin treatment improves cell viability and reduces damage in auditory HEI-OC1 cells (So et al. 2007). Similarly, blocking IL-6 attenuates noise-induced cochlear inflammatory responses and hearing impairment in mice (Wakabayashi et al. 2010). Transcriptional signal transducer and activator 1 (STAT1) and STAT3 are members of the STAT family with roles in the regulation of cell survival and death, inflammation, pro-inflammatory cytokine synthesis, and oxidative stress (Butturini et al. 2018, 2020). It has been reported that STAT1 and STAT3 play roles in auditory cell damage but with opposite effects (Bhatta et al. 2019; Borse et al. 2017; Jiang et al. 2016; Kaur et al. 2016; Levano and Bodmer 2015). STAT1 promotes inflammation and apoptosis in cochleae, whereas STAT3 acts as a pro-survival molecule that inhibits apoptosis and contributes to the resolution of inflammation. Advances in the pharmacology and molecular biology of hearing that target the molecular mechanisms of cisplatin-mediated ototoxicity provide strategies for finding new agents that prevent hearing loss (Febles et al. 2022; Nan et al. 2022). The most striking finding in this regard is the FDA's approval of sodium thiosulfate in 2022 in the USA for the prevention of hearing loss in pediatric patients with localized non-metastatic solid tumors (Dhillon 2023). However, there is still a need to develop more ear protection agents that are effective in preventing cisplatin-induced ototoxicity.

Apelin-13 is the most biologically active isoform of apelin and the ligand for the G protein-coupled receptor APJ (Habata et al. 1999). An increasing body of evidence suggests that apelin-13 regulates cell proliferation, differentiation, and apoptosis, and is a potential protective molecule against a variety of diseases (Falcao-Pires and Leite-Moreira 2005). Apelin-13 protects against injury in the brain, heart, kidney, and lungs, and these effects are associated with its anti-inflammatory, antioxidant, and anti-apoptotic bioactivities (Bircan et al. 2016; Ishimaru et al. 2017; Xia et al. 2021; Zhou et al. 2016). Apelin-13 mediates neuroprotective effects by inhibiting neuroinflammation, reducing brain damage, and modulating memory (Shen et al. 2022; Wan et al. 2022). Exogenous apelin-13 attenuates early brain injury induced by subarachnoid hemorrhage by inhibiting neuroinflammation and endoplasmic reticulum stress-mediated oxidative stress, and ameliorates LPS-induced microglial neuroinflammation via inhibiting STAT3 signaling pathway (Xu et al. 2019; Zhou et al. 2019). In myocardial cells, apelin-13 protects against ischemia–reperfusion injury by suppressing oxidative stress and apoptosis, and attenuates cisplatin-induced cardiomyocyte cytotoxicity by inhibiting ROS-mediated DNA damage (Yang et al. 2015; Zhang et al. 2017). In our previous study in vitro, apelin-13 protected HEI-OC1 cells from cisplatin-induced cytotoxicity by inhibiting apoptosis (Yin et al. 2020). However, few in vivo studies have investigated the role of apelin-13 in cisplatin-induced ototoxicity. In this study, we investigated the effect of apelin-13 on cisplatin-induced ototoxicity in mice and elucidated the potential molecular mechanisms underlying it.

Materials and methods

Major reagents and animals

Cisplatin (Sigma Co.) and apelin-13 powders (Phoenix Pharmaceuticals Co.) were dissolved in solvent. TUNEL kits (Life Technologies, Invitrogen), 2´,7´-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma Technologies) and JC-1 assay kits (Jiancheng Institute of Biology) were used following the manufacturers' instructions. C57BL/6 mice with normal hearing (Jinan Pengyue Experimental Co.) were housed in an SPF-class animal room with temperature and circadian rhythm control. The animal studies were conducted following the requirements of the protocol of the Jining Medical University Animal Care Committee (SYXK-Shandong province-2018–0002).

Animal and cultured cochlear explants experiments

30-day-old male C57BL/6 mice were randomly assigned to the control, cisplatin, apelin-13, or apelin-13 plus cisplatin groups. The doses and treatment duration of apelin-13 and cisplatin given by intraperitoneal injection were the same as those used in previous studies (Khoshsirat et al. 2021; Yu et al. 2019). Mice in the cisplatin group received 3 mg/

kg cisplatin for 7 days, and those in the apelin-13 group were given 100 μg/kg apelin-13 for 7 days. The mice in the combination group received apelin-13 2 h before the cisplatin injection. Physiological saline served as the control. In in vitro experiment, the middle-turn cochlear explants acquired from 3- to 4-day-old mice were cultured and randomly divided into four groups, and then treated with apelin-13 and/or cisplatin as described previously (Yin et al. 2020). Cochlear explants were incubated with DMEM medium containing either 30 μM cisplatin or 10 nM apelin-13 for 24 h or were pretreated with apelin-13 for 2 h, and then treated with cisplatin for 24 h. Cochlear explants without any treatment served as controls.

Hearing threshold test by auditory brainstem response (ABR)

The auditory brainstem response (ABR) test measured the auditory thresholds of mice in each group 1 day before and 7 days after injection. A computer-aided evoked potential system (Intelligent Hearing Systems) was used to record mouse hearing thresholds. Mice were anesthetized with pentobarbital sodium (50 mg/kg) and placed on a warming pad in a closed, soundproof audiometric chamber. The recording electrode was inserted into the subcutaneous tissue at the top of the mouse skull, and the reference electrode and the ground electrode were placed in the ipsilateral mastoid subcutaneous tissue and the dorsal subcutaneous tissue, respectively. The pure-tone pulse stimulus intensities at 8 kHz, 16 kHz, and 32 kHz were started at 90 dB and were decreased by 5 dB steps until no waves were detected, and the lowest stimulus intensity at each frequency was recorded as the hearing threshold.

Preparation of cochleae sections

Mice were euthanized by anesthesia after the experiments. The cochleae were rapidly dissected from the head and placed in 4% paraformaldehyde at 4 °C for 24 h, placed in 10% EDTA solution for decalcification until the cochlea became translucent and then dehydrated in a gradient of 10%, 20%, and 30% sucrose solution. Finally, they were embedded in OCT compound (Tissue-Tek, Sakura Finetek) and sliced into 5 μm sections using a cryostat.

HE, immunofluorescence, and immunohistochemistry staining

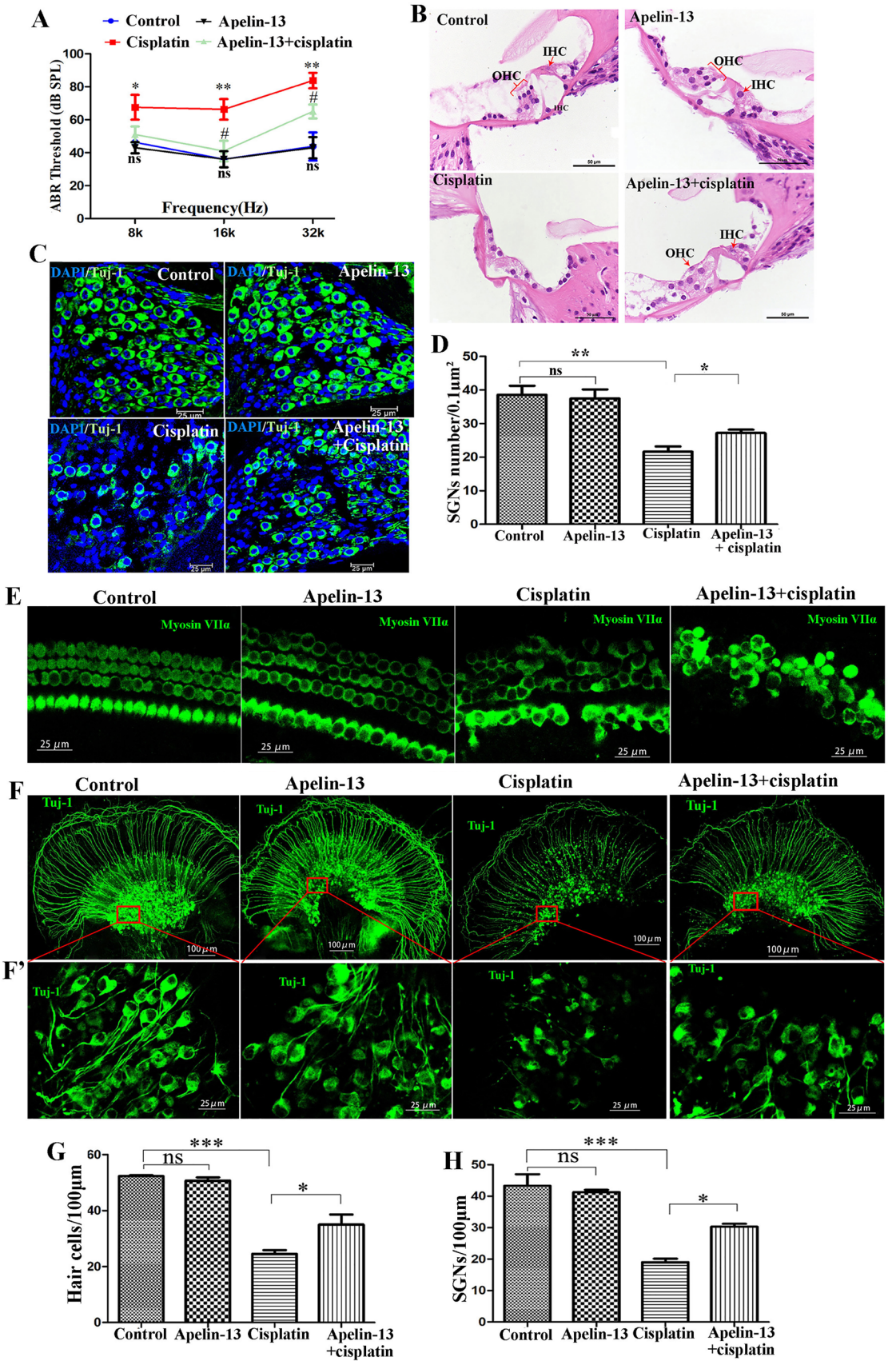
Cochlear cryosections were stained with hematoxylin–eosin (HE) in a conventional automated staining apparatus and then observed and photographed with a light microscope to assess histological changes in the cochleae. The immunofluorescence staining procedure for cochlear cryosections and explants was as follows: cochlear cryosections and fixed cochlear explants were washed three times with PBS and permeabilized with 0.2% Triton-X 100 in PBS for 10 min. After blocking antigens with 1% BSA in PBS, the specimens were incubated with primary antibodies for more than 24 h at 4 °C. After washing with PBS, the specimens were incubated with secondary antibodies and 4′ 6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma) for 1 h at room temperature in the dark. Finally, the specimens were observed and photographed using a confocal microscope system (LEICA TSC-SP8). Immunohistochemical staining of cochlear sections was carried out following the protocol of the SAP detection kit (SAP-9100, Zhongshan Jinqiao). Briefly, after permeabilization and antigen repair, specimens were blocked with goat serum for 1 h at room temperature and then incubated with primary antibodies for more than 24 h at 4 °C. After washing, the specimens were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and were treated with DAB horseradish peroxidase color development kit. Specimens were observed under a light microscope. The primary and secondary antibodies used in these experiments were as follows. Mouse anti-myosin VIIα (#138-1,1:500, Developmental Studies Hybridoma Bank), rabbit anti-p-STAT3 (#9145, 1:500), rabbit anti-p-STAT1 (#9177, 1:500), and rabbit anti-cleaved-caspase 3 (#9664S, 1:800) were purchased from Cell Signaling Technology. Rabbit anti-IL-6 (#GB11117, 1:800) and rabbit anti-TNF-α (#GB11188,1:200) were purchased from Servicebio Technology. Mouse anti-TuJ-1 (#MAB1195, 1:500) was purchased from Novus Bio. The secondary immunofluorescent antibodies, Alexa Flour-488 and Alexa Flour-546, were purchased from Invitrogen.

Semi-quantitative immunohistochemical analysis

A semi-quantitative scoring system of immunohistochemical staining was used to evaluate the expression level of TNF-α and IL-6. Staining intensity was quantitatively assessed as 0 scores (negative), 1 score (weak positive), 2 scores (moderate positive), and 3 scores (strong positive). The percentages of HCs and SGNs at each of the four staining intensities were counted respectively. The final score was obtained by multiplying the percentage of the HCs or SGNs with different staining intensities by the corresponding staining intensity score. Final scores = 0 × negative percentage + 1 × weak positive percentage + 2 × moderate positive percentage + 3 × strong positive percentage.

TUNEL assay for apoptosis detection

Apoptotic cells were detected in cochlear explants and cryosections using Click-iT Plus TUNEL assay kits as described previously (Yin et al. 2017). The specimens were


A
Control
Apelin-13
Cisplatin
Apelin-13+cisplatin
ABR Threshold (dB SPL)
Frequency(Hz)
8k
16k
32k
B
Control
Apelin-13
Cisplatin
Apelin-13+cisplatin
IHC
OHC
C
DAPI/Tuj-1
Control
Apelin-13
Cisplatin
Apelin-13 +Cisplatin
D
SGNs number/0.1μm²
Control
Apelin-13
Cisplatin
Apelin-13 + cisplatin
E
Myosin VIIα
F
Tuj-1
F'
G
Hair cells/100μm
H
SGNs/100μm

◂**Fig. 1** Apelin-13 protected against cisplatin-induced hearing loss and damage to cochlear HCs and SGNs in mice and cultured cochlear tissue. **A** ABR thresholds of mice in the control, apelin-13, cisplatin, and apelin-13 plus cisplatin groups. $^{*}P<0.05$, $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$ vs. cisplatin group, ns means no significance between control and apelin-13 groups, $n=4$. **B** HE staining of HCs (IHC, inner hair cell; OHC, outer hair cell) in the middle turns of cochlea. **C** Immunofluorescence staining of cochlear SGNs labeled by TuJ-1 (green). Nuclei were stained with DAPI. **D** Quantitative analysis of SGNs cell counts. $^{*}P<0.05$, $^{**}P<0.01$, *ns* no significance, $n=3$. **E** Immunofluorescence staining of HCs labeled by myosin VIIa (green) in cultured cochlear explants. Scale bar = 25 μm. **F**–**F´** Immunofluorescent images of SGNs labeled with TuJ-1 (green) in cochlear explants. **G** Quantification of surviving HC counts in the cochlear middle turns within a 100 μm region ($n=4$). **H** Quantification of the average number of surviving SGNs in the middle turns within a 0.01 mm^2 area ($n=4$). $^{*}P<0.05$, $^{***}P<0.001$, *ns* not significant

then observed and photographed using a LEICA confocal microscopy system.

Protein extraction and immunoblotting experiments

The collected cochlear tissue was washed with cold PBS, lysed in RIPA buffer (#P0013B, Beyotime Institute of Biotechnology) on ice for 0.5 h. After low-temperature centrifugation of the samples, the total protein was extracted. Equal masses of protein were added to 4–12% SDS-PAGE gels, separated by electrophoresis, and transferred to polyvinylidene difluoride membranes (PVDF, #IPVH00010, Immobilon-P). The PVDF membranes were placed in TBST buffer containing 5% skim milk at room temperature for 2 h followed by incubation with primary antibodies at 4 °C overnight, then were incubated with the secondary antibodies for 1 h at room temperature after washing off primary antibodies. Protein intensities were detected by an ECL chemiluminescent solution and analyzed by ImageJ software. The antibodies used in this experiment were as follows. Rabbit anti-cleaved-caspase 3 (#9664S, 1:1000), mouse anti-Bcl-2 (#15071, 1:1000), rabbit anti-P-STAT3 (#9145, 1:1000), rabbit anti-P-STAT1 (#9177,1:1000), mouse anti-STAT3 (#9139, 1:1000), and rabbit anti-STAT1 (#14994,1:1000) were purchased from Cell Signaling Technology. Mouse anti-β-actin (#TA-09, 1:2000), horseradish-conjugated goat antirabbit IgG (#ZB-2301, 1:5000), and goat anti-mouse IgG (#ZB-2305, 1:5000) were purchased from ZSGB Biotechnology.

Measurement of mitochondrial membrane potential and ROS

Cochlear explants were incubated with 1X JC-1(#C2006, Beyotime Institute of Biotechnology) in serum-free DMEM medium for 20 min in a 37 °C incubator to detect the mitochondrial membrane potential in cultured cochlear explants. Cochlear explants were incubated with 10 μM DCFH-DA (#D6883, Sigma Technologies) in serum-free DMEM for 20 min at 37 °C to detect ROS levels in cochlear explants. After washing with buffer, the specimens were observed using a fluorescent microscope (Nikon ECLIPSE Ti2) under light-protected conditions.

ELISA for IL-6 and TNF-α detection

Levels of the cytokines IL-6 and TNF-α in the supernatant of cultured cochlear explants were measured using ELISA kits (DAKEWE, Biotech Co.) according to the manufacturer's protocol, and the absorbance values were measured with a microplate reader (Varioskan LUX). The concentrations of IL-6 and TNF-α were calculated according to a standard concentration curve.

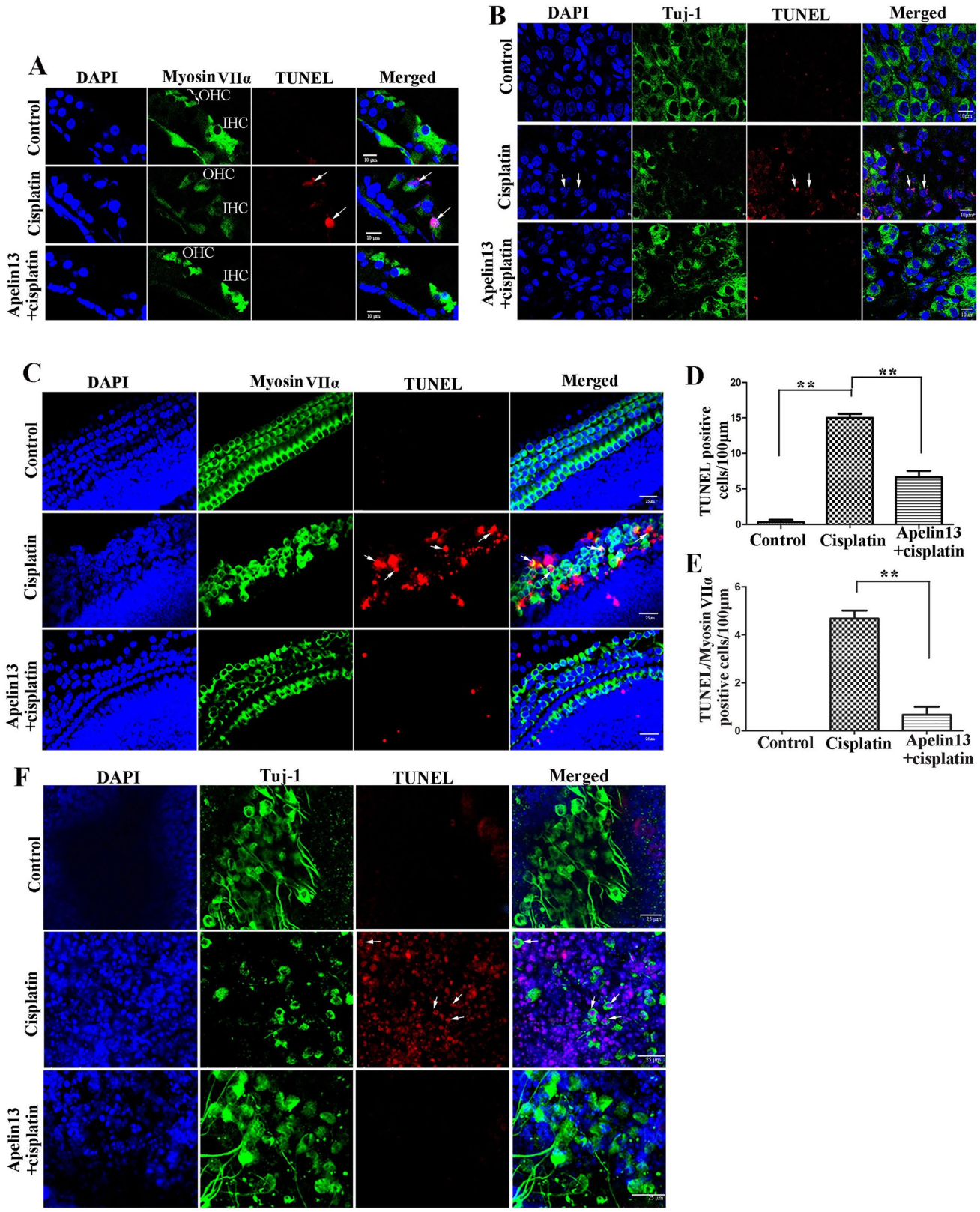
Statistical analyses

All data presented as mean ± S.E.M. were from at least three independent experimental replicates and were statistically analyzed using GraphPad Prism 6 software. Data conforming to the homogeneity of variance were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Data not conforming to the homogeneity of variance were analyzed by the non-parametric Kruskal–Wallis test followed by Dunn's test. $P<0.05$ was considered a significant difference.

Results

Apelin-13 protected against cisplatin-induced hearing loss and damage to cochlear HCs and SGNs in mice and in cultured cochlear explants

The role of apelin-13 in cisplatin-induced hearing loss was assessed by ABR testing of hearing thresholds. ABR thresholds at 8 kHz, 16 kHz, and 32 kHz were significantly elevated in mice injected intraperitoneally with cisplatin, indicating that cisplatin impaired auditory function and caused hearing loss in mice. Intraperitoneal injection with apelin-13 significantly reduced the cisplatin-induced increase in ABR thresholds, indicating that apelin-13 protected mice from cisplatin-induced hearing loss. The ABR thresholds of mice injected with apelin-13 alone were similar to those of controls, implying that apelin-13 does not affect the auditory function of non-treated mice (Fig. 1A). HE staining data showed that the structure of Corti was intact in the control and apelin-13 groups, indicated by an arrangement of three rows of outer HCs and one row of inner HCs. By contrast, the structure of Corti was severely



damaged, and HCs were absent in the cisplatin group. In the apelin plus cisplatin group, the structure of Corti was slightly damaged; some HCs were still present in this group (Fig. 1B). Immunostaining data showed the SGNs were full and evenly distributed in the control and apelin-13 groups, whereas there were fewer SGNs in the cisplatin group, and

◂**Fig. 2** Apelin-13 inhibited cisplatin-mediated apoptosis of HCs and SGNs in mouse cochlea and cultured cochlear explants. **A** TUNEL staining (red) images of apoptotic HCs (green, labeled by myosin VIIa) in mice. **B** TUNEL staining (red) images of apoptotic SGNs (green, labeled by TuJ-1) in mice. **C** TUNEL staining (red) images of apoptotic HCs (green, labeled by myosin VIIa) in cochlear explants. **D**, **E** Cell counts of TUNEL-positive cells and TUNEL-positive HCs in cochlear explants. **F** TUNEL staining (red) images of apoptotic SGNs (green, labeled by Tuj-1) in cochlear explants

those were unevenly distributed and had small cell bodies. The morphology of SGNs was improved in the apelin-13 plus cisplatin group compared with the cisplatin group, and there was a greater number of surviving SGNs (Fig. 1C, D). In experiments with cultured cochlear explants, the four rows of HCs were intact and well arranged in the control and apelin-13 groups, whereas the morphology of HCs was disrupted and disordered in the cisplatin group, and the number of surviving HCs was significantly lower (Fig. 1E, G). The morphology of HCs was improved in the cisplatin plus apelin-13 group compared with the cisplatin group, and the number of surviving HCs was significantly higher (Fig. 1E, G). Cisplatin also disrupted the morphology of SGNs and auditory nerve fibers. Cisplatin decreased the density of SGNs and resulted in thinner and fewer auditory nerve fibers connecting SGNs and HCs. Apelin-13 attenuated cisplatin-induced morphology disruption and increased the number of surviving SGNs and nerve fibers (Fig. 2F, F´, H). These in vitro and in vivo results suggested that apelin-13 exerted an otoprotective effect in cisplatin-induced hearing loss by attenuating cochlear HC and SGN damage.

Apelin-13 inhibited cisplatin-induced apoptosis of HCs and SGNs in mice cochleae and cultured cochlear explants

The apoptosis of HCs and SGNs in mice and cochlear explants was detected by TUNEL staining. There was no apparent TUNEL-positive staining of HCs and SGNs in control mice (Fig. 2A, B). The cisplatin group showed intense positive TUNEL staining. The level of staining was attenuated in the apelin-13 plus cisplatin group (Fig. 2A, B). Apoptotic cells in cochlear explants were also examined. No apoptotic cells were evident in the control group, whereas there were many apoptotic cells, especially in HCs and SGNs, in the cisplatin group. The number of TUNEL-positive cells was smaller in the apelin-13 plus cisplatin group (Fig. 2C, F). Cell-count analysis showed that the total number of apoptotic cells and apoptotic HCs was significantly smaller in the apelin-13 plus cisplatin group than in the cisplatin group (Fig. 2D, E). These results suggested that apelin-13 attenuated cisplatin-induced injury to HCs and SGNs by inhibiting apoptosis.

Apelin-13 inhibited cisplatin-induced apoptosis mediated by caspase 3 in HCs and SGNs in mice and cultured cochlear explants

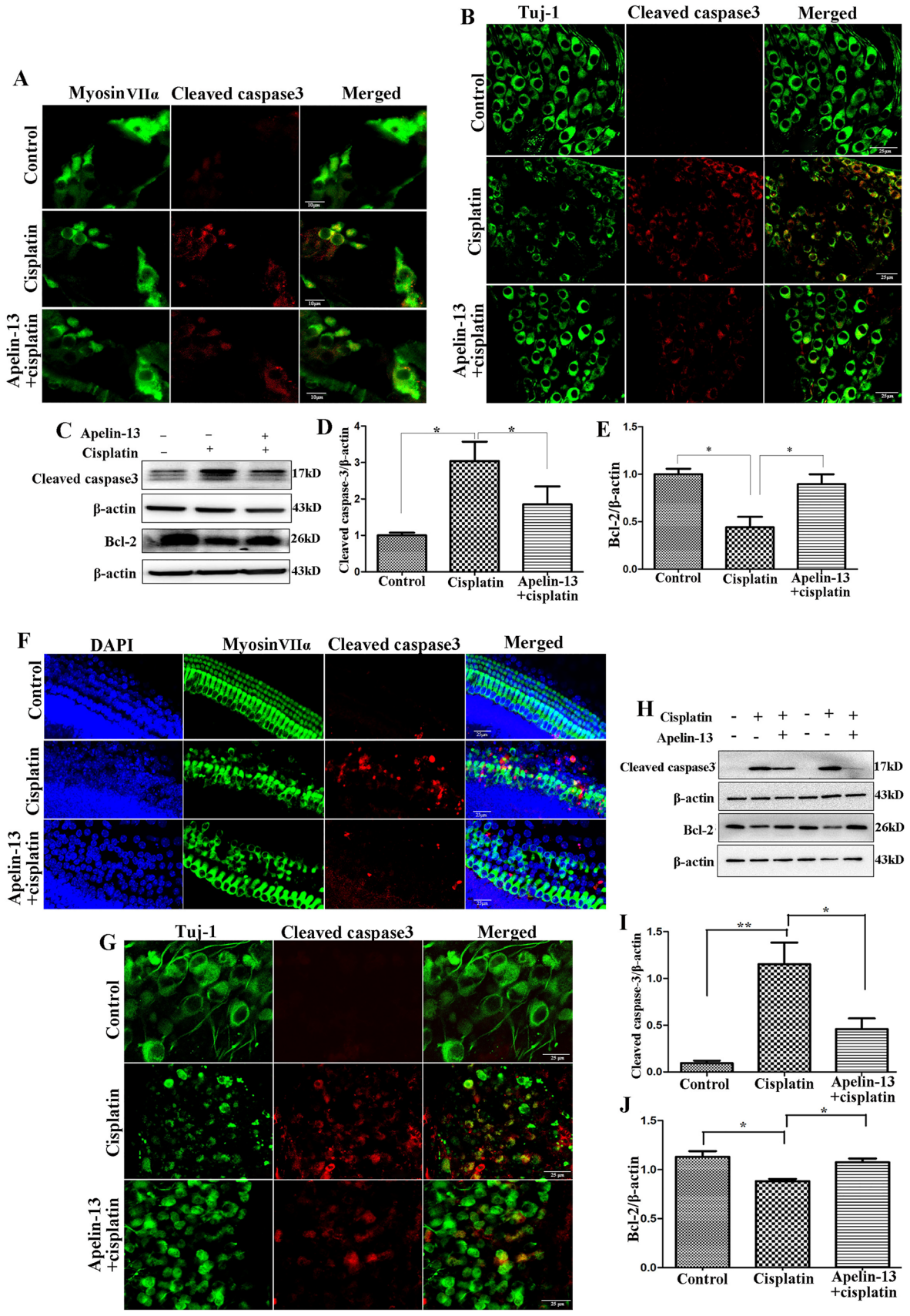
No cells marked by staining of cleaved caspase 3 were evident in HCs and SGNs from control mice or control cultured cochlear explants (Fig. 3A, B, F, G). The level of cleaved caspase 3 staining was higher in the cytoplasm of HCs and SGNs from mice cochleae and cultured cochlear explants in the cisplatin groups than in the apelin-13 plus cisplatin groups (Fig. 3A, B, F, G). Western blotting results confirmed that apelin-13 significantly decreased the cisplatin-induced increase in cleaved caspase 3 expression in mice cochleae and cultured cochlear explants, but increased protein levels of the anti-apoptotic protein Bcl-2 (Fig. 3C, D, E H, I, G). These results suggested that apelin-13 attenuated cisplatin-induced apoptosis in HCs and SGNs in the cochleae by inhibiting the caspase 3-dependent apoptotic pathway.

Apelin-13 prevented mitochondrial dysfunction and oxidative stress caused by cisplatin in vivo and in vitro

We investigated the mitochondrial membrane potential using JC-1 staining in cochlear explants. In control cochlear explants, JC-1 aggregates showed noticeable red fluorescence, indicating the mitochondria were intact. By contrast, in cisplatin-treated cochlear explants, JC-1 monomers showed green fluorescence, indicating that the mitochondrial membrane potential was disrupted (Fig. 4A). Cochlear explants from the apelin-13 plus cisplatin group had less green fluorescence and more red fluorescence than the cisplatin group (Fig. 4A). This result suggested that apelin-13 protected against cisplatin-induced mitochondrial dysfunction in cochlear cells. It is known that mitochondrial dysfunction affects the generation of ROS and oxidative stress. Cochlear explants from the cisplatin group had stronger DCFH-DA green fluorescence (indicating high levels of ROS) than the apelin-13 plus group (Fig. 4B). In vivo, apelin-13 significantly reduced the cisplatin-induced increase in malondialdehyde (MDA) levels, which are another indicator of cisplatin-induced oxidative stress, in mouse cochleae (Fig. 4 C).

Apelin-13 inhibited cisplatin-induced expression of TNF-α and IL-6 in HCs and SGNs in vivo and cultured cochlear explants

As shown in Fig. 5A, ELISA data showed that apelin-13 significantly decreased the cisplatin-induced increase in TNF-α and IL-6 levels in the supernatant from cultured cochlear explants. Immunohistochemical staining showed that TNF-α and IL-6 staining was more frequently diffuse in HCs and


A
MyosinVIIα
Cleaved caspase3
Merged
Control
Cisplatin
Apelin-13 +cisplatin
B
Tuj-1
Cleaved caspase3
Merged
Control
Cisplatin
Apelin-13 +cisplatin
C
Apelin-13
Cisplatin
Cleaved caspase3
17kD
β-actin
43kD
Bcl-2
26kD
β-actin
43kD
D
Cleaved caspase-3/β-actin
Control
Cisplatin
Apelin-13 +cisplatin
E
Bcl-2/β-actin
Control
Cisplatin
Apelin-13 +cisplatin
F
DAPI
MyosinVIIα
Cleaved caspase3
Merged
Control
Cisplatin
Apelin-13 +cisplatin
G
Tuj-1
Cleaved caspase3
Merged
Control
Cisplatin
Apelin-13 +cisplatin
H
Cisplatin
Apelin-13
Cleaved caspase3
17kD
β-actin
43kD
Bcl-2
26kD
β-actin
43kD
I
Cleaved caspase-3/β-actin
Control
Cisplatin
Apelin-13 +cisplatin
J
Bcl-2/β-actin
Control
Cisplatin
Apelin-13 +cisplatin

◂**Fig. 3** Apelin-13 inhibited caspase 3 expression in HCs and SGNs in mice and cultured cochlear explants. **A** Immunofluorescent images of cleaved caspase 3 (red) in HCs (green) in mice. **B** Immunofluorescent images of cleaved caspase 3 (red) in SGNs (green) in mice. **C**–**E** Cleaved caspase 3 and Bcl-2 were assayed by western blotting in mouse cochleae. $^{*}P<0.05$, n = 3. **F** Immunofluorescent images of cleaved caspase 3 (red) in HCs (green) in cochlear explants. **G** Immunofluorescent images of cleaved caspase 3 (red) in SGNs (green) in cochlear explants. **H**–**J** Cleaved caspase 3 and Bcl-2 were assayed by western blotting in cochlear explants. $^{*}P<0.05$, $^{**}P<0.01$, $n=3$ (color figure online)

SGNs in the cisplatin group, and of the positive samples, the intensity of TNF-α and IL-6 always labeled strong or moderate in the samples. In the control and apelin-13 plus cisplatin group, TNF-α and IL-6 commonly showed limited expression in extent or weak expression intensity (Fig. 5B, E). The semi-quantitative immunohistochemical score showed that TNF-α and IL-6 expression was high in mouse cochlear HCs and SGNs in the cisplatin groups, and lower in the control and apelin-13 plus cisplatin groups (Fig. 5C, D, F,G). These results implied that apelin-13 attenuated cisplatin-induced increases in pro-inflammatory TNF-α and IL-6 levels in vitro and in vivo.

Apelin-13 reduced STAT1 phosphorylation in HCs and SGNs exposed to cisplatin in vivo and in vitro

We investigated the expression of phosphorylated STAT1 (p-STAT1) in HCs and SGNs in mice and cochlear explants by immunofluorescence staining and western blotting. The fluorescence intensity of p-STAT1 was weak in HCs and SGNs in control mice and control cultured explants (Fig. 6A, B, F, G). Strong fluorescence was observed in HCs and SGNs from the cisplatin groups, and nuclear translocation of P-STAT1 occurred, with expression in both the cytoplasm and nucleus (Fig. 6A, B, F, G). p-STAT1 fluorescence intensity was weaker in HCs and SGNs from the apelin-13 plus cisplatin group than in the cisplatin groups, especially in the nuclei (Fig. 6A, B, F, G). Western blotting data showed that cisplatin significantly upregulated p-STAT1 levels, but not STAT1 levels, in mouse cochleae and cultured cochlear explants (Fig. 6C, D, E, H, I, J). Apelin-13 significantly decreased p-STAT1 levels in cisplatin-treated cochlear explants but did not significantly affect p-STAT1 levels in the cochleae of cisplatin-treated mice (Fig. 6C, D, E, H, I, J). These results suggested that apelin-13 prevented cisplatin-induced damage to HCs and SGNs by inhibiting STAT1 phosphorylation and nuclear translocation.

Apelin-13 increased STAT3 expression and phosphorylation in HCs and SGNs exposed to cisplatin in vivo and in vitro

The expression of phosphorylated STAT3 (p-STAT3) in HCs and SGNs in mice and cochlear explants was detected by immunofluorescence staining and western blotting. Immunofluorescence staining showed that p-STAT3 was expressed in the cytoplasm of HCs and SGNs from control groups and was significantly lower in HCs and SGNs from the cisplatin group. Cytoplasmic p-STAT3 expression was significantly stronger in HCs and SGNs from the apelin-13 plus cisplatin group than those from cisplatin group, and nuclear translocation was not evident. (Fig. 7A, B, F, G). Western blotting data showed that cisplatin significantly downregulated the STAT3 expression in the cultured cochlear explants and p-STAT3 levels in both mouse cochleae and cultured cochlear explants, and apelin-13 reversed these effects (Fig. 7C, D, E, H, I, J). These results suggested that apelin-13 can ameliorate the effects of cisplatin on STAT3 expression and phosphorylation in HCs and SGNs from mice and cochlear explants.

Discussion

Ototoxicity is one of the most serious adverse effects in cancer patients treated with cisplatin, with a high incidence and difficult to cure once it occurs. Cisplatin-induced ototoxicity is attributed to damage to the cochlear HCs and SGNs resulting in impaired sound perception and conduction (Wu et al. 2017). Recent studies show that apelin-13 is a protective peptide that modulates various physiological and pathological processes and is a potential therapeutic agent (Falcao-Pires and Leite-Moreira 2005; Huang et al. 2018). In this study, we found that apelin-13 had an otoprotective effect against cisplatin-induced ototoxicity in a mouse model. Intraperitoneal injection of apelin-13 before cisplatin treatment reduced the cisplatin-induced elevation of ABR thresholds at 8 kHz, 16 kHz, and 32 kHz, indicating that apelin-13 protected mice from cisplatin-induced hearing loss across a wide range of frequencies. Apelin-13 treatment alone did not affect hearing thresholds or morphology of HCs and SGNs in mice, but ameliorated cisplatin-elicited hearing thresholds and damage to cochlear HCs and SGNs in vitro and in vivo. These results suggested that apelin-13 protects hearing by preventing cisplatin-induced damage of HCs and SGNs.

Apoptosis of HCs and SGNs in cochleae is one of the primary mechanisms underlying cisplatin-induced ototoxicity. Apelin-13 has protective effects in several diseases by inhibiting apoptosis through different signaling pathways (Liu et al. 2017). In the current work, apelin-13

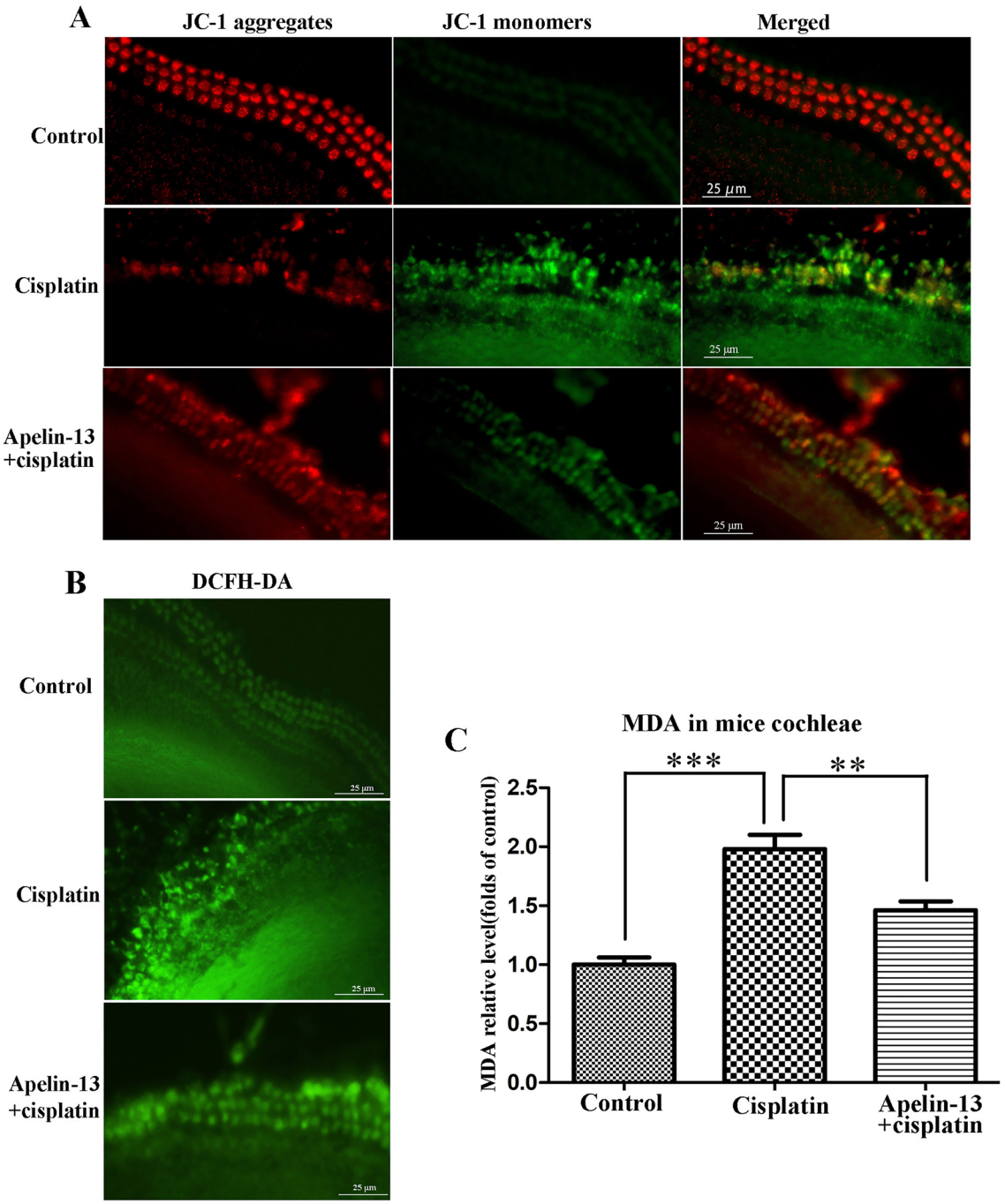


Fig. 4 Apelin-13 attenuated cisplatin-induced mitochondrial membrane potential dysfunction, generation of ROS and MDA. **A** JC-1 aggregates showed red fluorescence in mitochondria from control cochlear explants, indicating intact mitochondria. When the mitochondrial membrane potential is disrupted (for example, in the presence of cisplatin), JC-1 monomers showed green fluorescence. **B** ROS generation was detected by DCFH-DA staining (green) in cochlear explants. **C** MDA levels in whole cochleae. $^{**}P<0.05$, $^{***}P<0.001$, $n=4$ (color figure online)

reduced the number of apoptotic HCs and SGNs in cochlear explants and the cochleae of mice exposed to cisplatin, indicating that apelin-13 may attenuate HCs and SGNs damage by inhibiting apoptosis. Previous studies demonstrated that cisplatin triggers apoptosis of HCs and SGNs mainly by apoptotic pathways involving caspases 3 and Bcl-2 family members (Wang et al. 2022). Cleaved caspase 3 is the crucial effector enzyme in apoptosis. Apelin-13 reduced cisplatin-induced cleaved caspase 3 upregulation in HCs and SGNs in mice cochleae and cultured

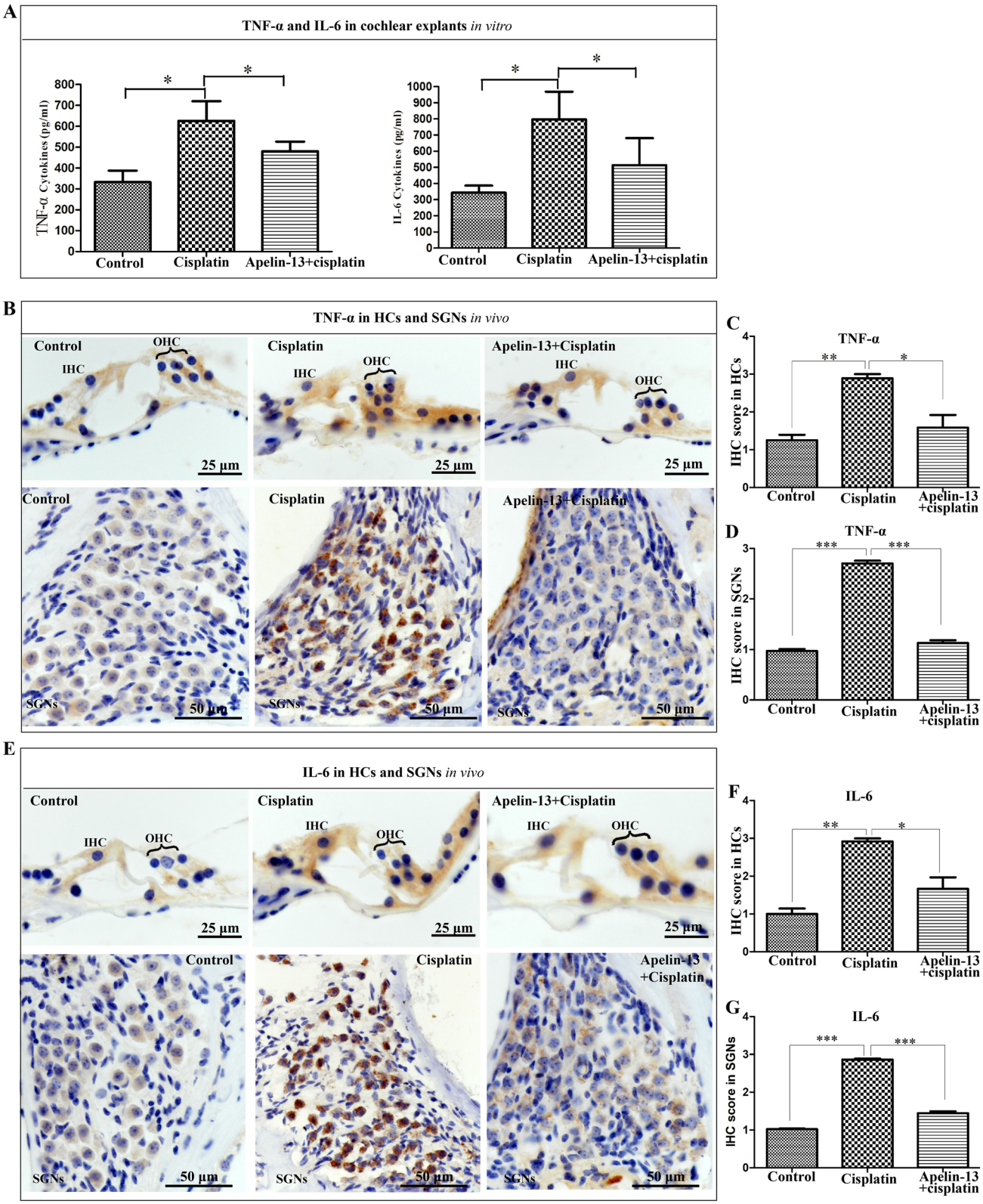
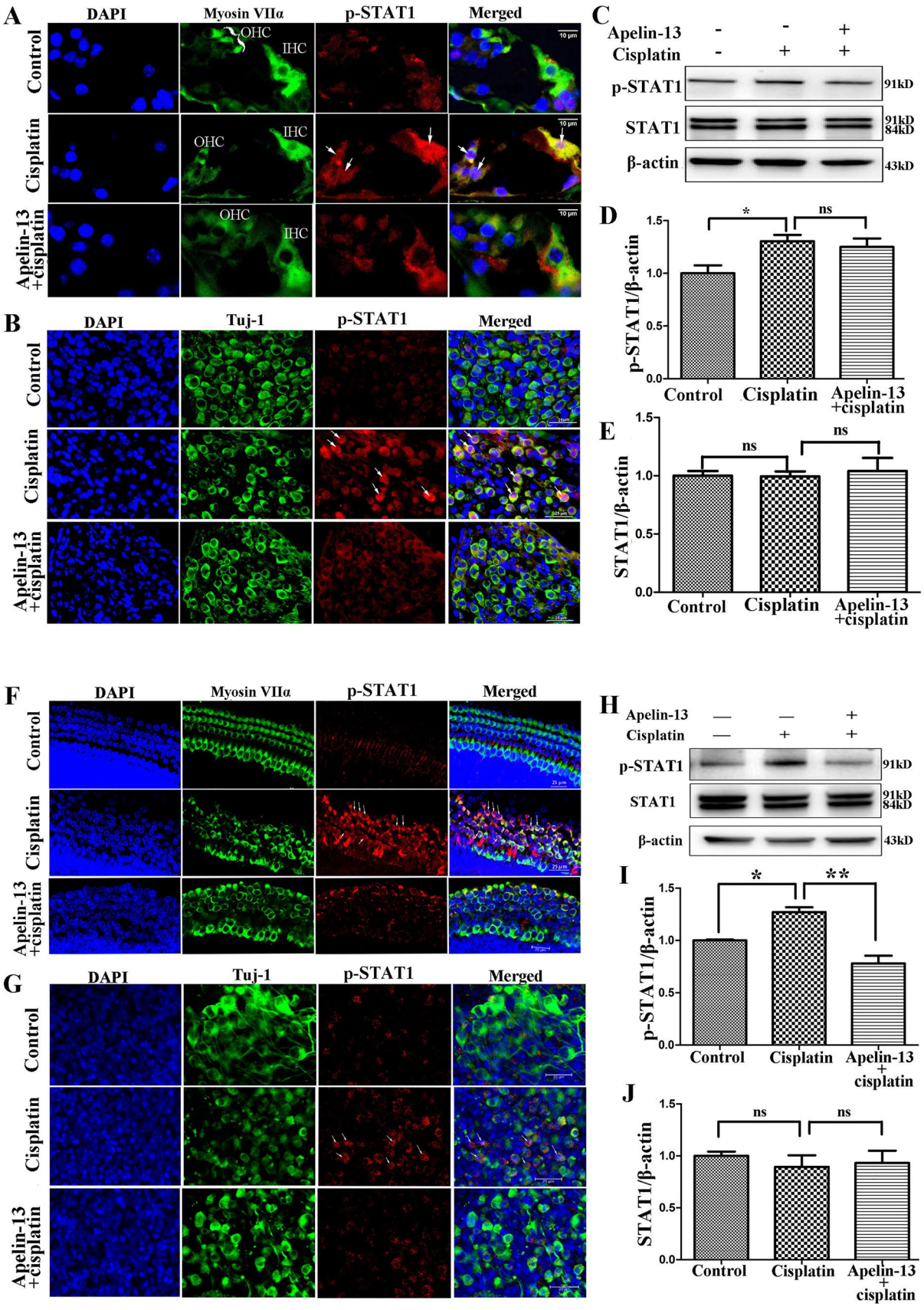


Fig. 5 Apelin-13 alleviated cisplatin-induced increases in TNF-α and IL-6 expression in cultured cochlear explants and in mice HCs and SGNs. **A** ELISA data of TNF-α and IL-6 release in the supernatants from cultured cochlear explants. $^{*}P<0.05$, $n=4$. **B** Representative immunohistochemical staining of TNF-α in HCs and SGNs. **C, D** IHC score of TNF-α expression in samples shown in B. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ **E** Representative immunohistochemistry staining of IL-6 in HCs and SGNs. **F, G** IHC score of IL-6 expression in samples shown in E. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$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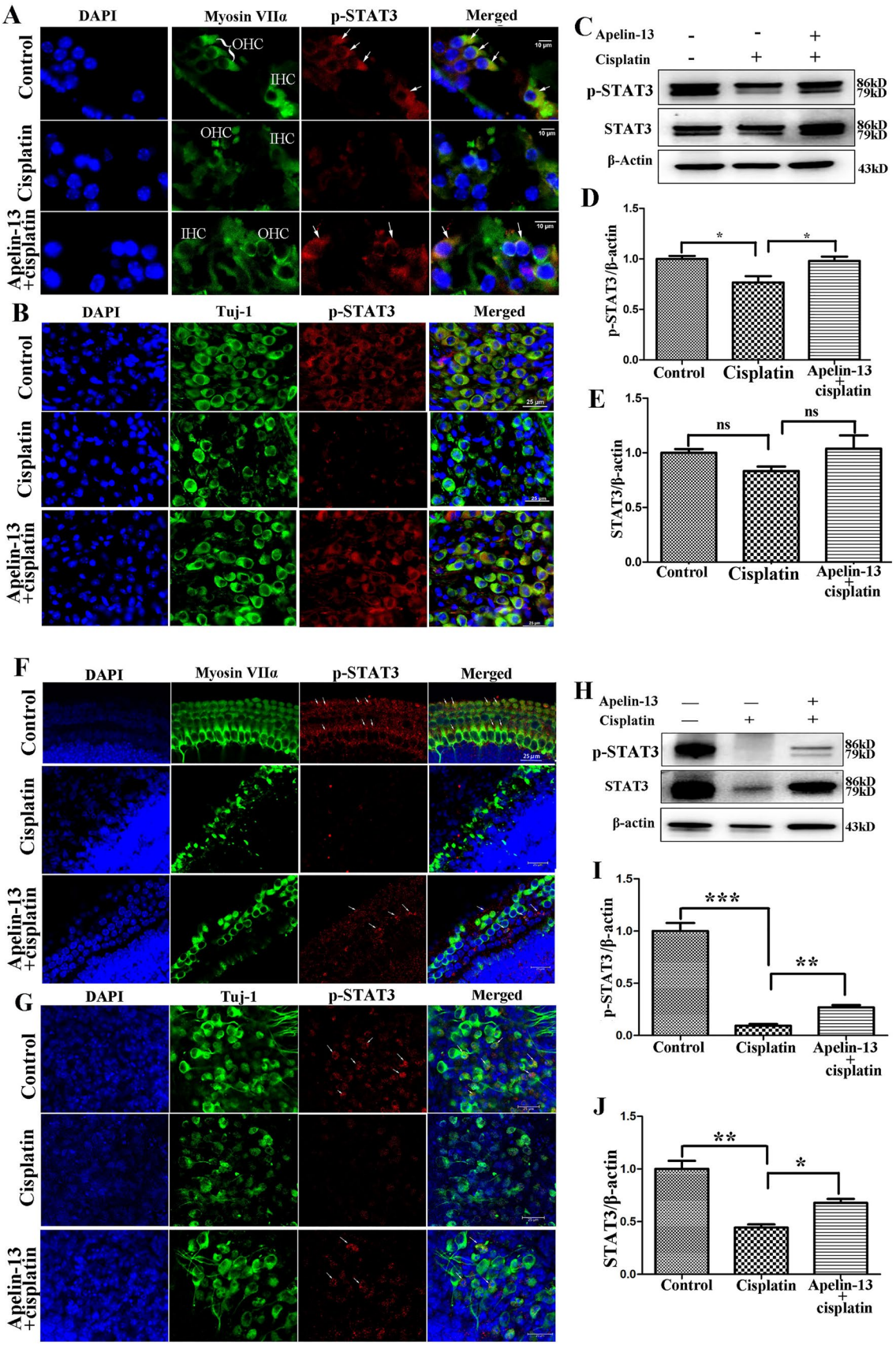
◂**Fig. 6** The effect of apelin-13 on STAT1 phosphorylation in HCs and SGNs in mouse cochleae and cultured cochlear explants. **A** p-STAT1 (red) staining in HCs (green) in mouse cochleae. **B** p-STAT1 (red) staining in SGNs (green) in mouse cochleae. **C** p-STAT1 and STAT1 expression in mouse cochleae assayed by western blotting. **D, E** Quantification of p-STAT1 and STAT1 levels in mouse cochleae analyzed by ImageJ software. $^{*}P<0.05$, *ns* no significance, $n=3$. **F** p-STAT1 (red) staining in HCs (green) in cultured cochlear explants. **G** p-STAT1 (red) staining in SGNs (green) in cultured cochlear explants. **H** p-STAT1 and STAT1 expression in cultured cochlear explants assayed by western blotting. **I, G** Quantification of p-STAT1 and STAT1 levels in cultured cochlear explants analyzed by ImageJ software. $^{*}P<0.05$, $^{**}P<0.01$, *ns* no significance, $n=3$ (color figure online)

cochlear explants but reversed Bcl-2 downregulation. This finding is partially consistent with a previous report of the effects of apelin-13 on hair cell-like cells in an in vitro study (Niknazar et al. 2019). These data suggested that apelin-13 prevented the caspase 3-dependent apoptosis pathway induced by cisplatin.

Mitochondria are critical signaling platforms for activating the apoptotic pathway and are the primary source of cellular ROS. Excessive ROS production is one of the important pathological mechanisms of cisplatin-induced ototoxicity (Kishimoto-Urata et al. 2022; Okur and Djalilian 2022). Studies have demonstrated that apelin-13 improves mitochondrial function and reduces ROS-mediated oxidative stress injury in cardiomyocytes and lungs (Ye et al. 2021; Zhang et al. 2019). In this study, apelin-13 prevented cisplatin-induced destruction of mitochondrial membrane potential and ROS generation in cochlear explants and prevented the cisplatin-induced increase in malondialdehyde levels in mouse cochleae. These results indicated that the protective effect of apelin-13 was related to improved mitochondrial function and antioxidative stress activity. Several studies suggest that ROS caused by cisplatin activates the inflammation in cochlea (Ramkumar et al. 2021), but other studies suggest that cisplatin may directly induce the activity of inflammatory factors that mediate the inflammatory response, triggering the production of ROS and ultimately causing cochlear damage (Gentilin et al. 2019). Studies in several models of auditory cell damage have shown that cisplatin elicits inflammation mostly by increasing the expression of the pro-inflammatory cytokines TNF-α and IL-6 (So et al. 2007). These studies imply that anti-inflammatory agents might treat hearing loss (Kalinec et al. 2017). Apelin-13 has anti-inflammatory effects in several diseases by inhibiting pro-inflammatory factors TNF-α and IL-6 activity (Shen et al. 2022; Xia et al. 2021). In this study, apelin-13 reduced the cisplatin-induced TNF-α and IL-6 release in cochlear explants, as well as the expression of TNF-α and IL-6 in HCs and SGNs in mice. These results suggest that the attenuation of cisplatin-induced ototoxicity by apelin-13 is possibly related to the inhibition of inflammation.

STAT1 and STAT3 transcription factors are phosphorylated to form dimers that enter the nucleus to activate signal transduction, which regulates the expression of target genes and biological processes. Although STAT1 and STAT3 can be activated in response to the same stimuli, they usually play opposing roles in cell signaling. Disrupting the balance between STAT1 and STAT3 activity changes cell fate from survival to apoptosis, or from a pro-inflammatory to an anti-inflammatory response (Bhatta et al. 2019; Levano and Bodmer 2015; Rosati et al. 2019). In several models of auditory cell damage and hearing loss, STAT1 promotes inflammation and apoptosis, whereas STAT3 has the opposite effect, acting as a pro-survival molecule that inhibits apoptosis and helps resolve inflammation (Kaur et al. 2011, 2016; Levano and Bodmer 2015). ROS can initiate an inflammatory and apoptotic cascade in the cochlea by activating STAT1, and loss of STAT1 in rats and mice protects against drug-induced ototoxicity (Jiang et al. 2016; Kaur et al. 2011; Schmitt et al. 2009). By contrast, STAT3 protects against oxidative stress induced by cisplatin and upregulates anti-apoptotic Bcl-xL and Bcl-2 expression in cells of Corti (Rosati et al. 2019). Several otoprotective agents attenuate the ototoxicity of cisplatin by upregulating the ratio of phosphorylation-activated STAT3/STAT1 in auditory cells (Bhatta et al. 2019; Borse et al. 2017; Levano and Bodmer 2015). In this study, cisplatin upregulated p-STAT1 expression and promoted its nuclear translocation while downregulating p-STAT3 expression in HCs and SGNs; therefore, cisplatin disrupted the balance between STAT1 and STAT3. These results suggest that cisplatin-induced ototoxicity is closely associated with the activation of STAT1 and inhibition of STAT3 in HCs and SGNs. This observation is consistent with previous reports investigating the mechanisms of cisplatin-induced ototoxicity in auditory cells in vivo and in vitro (Levano and Bodmer 2015; Rosati et al. 2019). In addition, our study showed that cisplatin not only inhibited the phosphorylation of STAT3 both in vivo and in vitro but also greatly reduced total STAT3 expression in cultured cochlear explants in vitro. Apelin-13 reduced the cisplatin-induced phosphorylation and activation of STAT1 in HCs and SGNs, indicating that apelin-13 attenuates cisplatin-induced ototoxicity by inhibiting STAT1 transcriptional activation in the nucleus. Although apelin-13 increased the phosphorylation of STAT3 in cisplatin-treated HCs and SGNs in vitro and in vivo, there was no observable nuclear translocation. This result indicates that apelin-13 does not modulate STAT3 transcriptional activation in cisplatin-induced ototoxicity. In addition to nuclear expression as a transcription factor, STAT3 can also function as a non-transcription factor, with activated STAT3 translocating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43kD
D
p-STAT3/β-actin
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Apelin-13 + cisplatin
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◂**Fig. 7** The effect of apelin-13 on STAT3 activation in HCs and SGNs from mouse cochleae and cultured cochlear explants. **A** Immunofluorescent images of p-STAT3 (red) staining in HCs (green) in mouse cochleae. **B** Immunofluorescent images of p-STAT3 (red) staining in SGNs (green) in mouse cochleae. **C** The expression level of p-STAT3 and STAT3 in mouse cochleae assayed by western blotting. **D, E** Quantification of p-STAT3 and STAT3 expression levels in mouse cochleae analyzed by ImageJ software. $^{*}P<0.05$, *ns* no significance, $n=3$. **F** Immunofluorescent images of p-STAT3 (red) staining in HCs (green) in cochlear explants. **G** Immunofluorescent images of p-STAT3 (red) staining in SGNs (green) in cochlear explants. **H** The expression level of p-STAT3 and STAT3 in cultured cochlear explants assayed by western blotting. **I, J** Quantification of p-STAT3 and STAT3 expression in cochlear explants analyzed by ImageJ software. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.05$, $n=3$ (color figure online)

to mitochondria, endoplasmic reticulum, and lysosomes where it can bind to proteins (Li et al. 2022). Therefore, we speculate that apelin-13 attenuates cisplatin ototoxicity by regulating the non-transcriptional function of STAT3. However, the specific molecular mechanisms of apelin-13 on STAT3 in cisplatin-induced ototoxicity need further investigation.

Conclusion

In summary, we prove that apelin-13, as a promising therapeutic agent, effectively prevented cisplatin-induced ototoxicity by protecting HCs and SGNs in vivo and in vitro. Mechanistic studies showed that apelin-13 prevented cisplatin-induced ototoxicity by inhibiting apoptosis, ROS production, TNF-α and IL-6 expression, and regulating STAT1 and STAT3 transcription factors phosphorylation in HCs and SGNs. However, the intricate relationship of their potential upstream or downstream regulatory roles warrant further exploration.

Author contributions HY: experimental design, methodology, writing—original draft. QY: writing—reviewing and editing. YS and BY: conducted experimental procedures, data collecting and analyzing. HZ and FW: software, investigation. YG, LZ, and WZ: conceptualization, data analyzing, validation.

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Availability of data The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

Declarations

Conflict of interest The authors declare they have no conflicts of interest.

Ethics approval This study was approved by the Medical Ethics Committee of Jining Medical University (2019-JC-001).

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