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ORIGINAL RESEARCH

The Upregulation of IL-1β Induced by Cisplatin Triggers PI3K/AKT/MMP9 Pathway in Pericytes Mediating the Leakage of the Blood Labyrinth Barrier

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Background: Blood–labyrinth barrier (BLB) damage has been recognized as a key mechanism underlying cisplatin (CDDP)-induced hearing loss. Inflammation within the cochlea, triggered by CDDP, is a key pathological response. However, the relationship between CDDP-induced inflammation and BLB dysfunction remains elusive.

Materials and Methods: In vivo and in vitro BLB models were used to explore the inflammatory mechanisms underlying CDDP ototoxicity. C57BL/6J mice were treated with CDDP and IL-1β levels, BLB permeability, and hearing thresholds were assessed using ELISA, histological staining, ABR test and BLB leakage tests. In vitro BLB models, the effect of IL-1β on MMP9 expression, PI3K-AKT pathway activation, and endothelial barrier permeability were examined via Western blot, TEER value test, and FITC extraction analysis. In addition, inhibitors of IL-1β, MMP9, and PI3K-AKT were used to analyze the specific mechanisms.

Results: After CDDP treatment, IL-1β upregulation in the stria vascularis disrupted tight junctions, increased BLB permeability, and led to hearing loss. Notably, IL-1β inhibition with AS101 attenuated hearing threshold elevation and BLB damage in CDDP-treated mice. Mechanistically, CDDP triggered IL-1β release from endothelial cells. IL-1β promoted MMP9 secretion from pericytes via the PI3K/AKT pathway, leading to disruption of tight junctions. Both MMP9 and PI3K-AKT inhibitors abrogated IL-1β-induced changes.

Conclusion: Our findings suggest that CDDP initiates a cascade of events starting with IL-1β release from endothelial cells. This release triggers the activation of PI3K/AKT pathway and upregulation of MMP9 expression in pericytes, which increases BLB permeability and leds to hearing loss. IL-1β and the PI3K-AKT pathway are promising therapeutic targets,offering hope for patients with CDDP-induced hearing loss.

Keywords: cisplatin, BLB, IL-1β, MMP9, PI3K/AKT pathway

Introduction

Cisplatin (CDDP), a pivotal chemotherapy drug for solid cancers, enhances patient survival and quality of life but is marred by ototoxicity, notably permanent hearing loss in up to 80% of patients.[1,2] Amidst the intensifying demographic shift towards an aging population, the incidence of cancer among elderly individuals has been steadily escalating. The ototoxicity associated with these malignancies poses a formidable clinical challenge specifically for this geriatric cohort. A retrospective analysis by Argiris et al[3] on head and neck cancer patients in the United States revealed that elderly individuals are more susceptible to CDDP-induced toxicity. In elderly populations, hearing loss not only causes communication barriers, social isolation, and depression, but also significantly impacts cognitive function,[4] mobility,[5] and overall quality of life.[6] It is closely associated with

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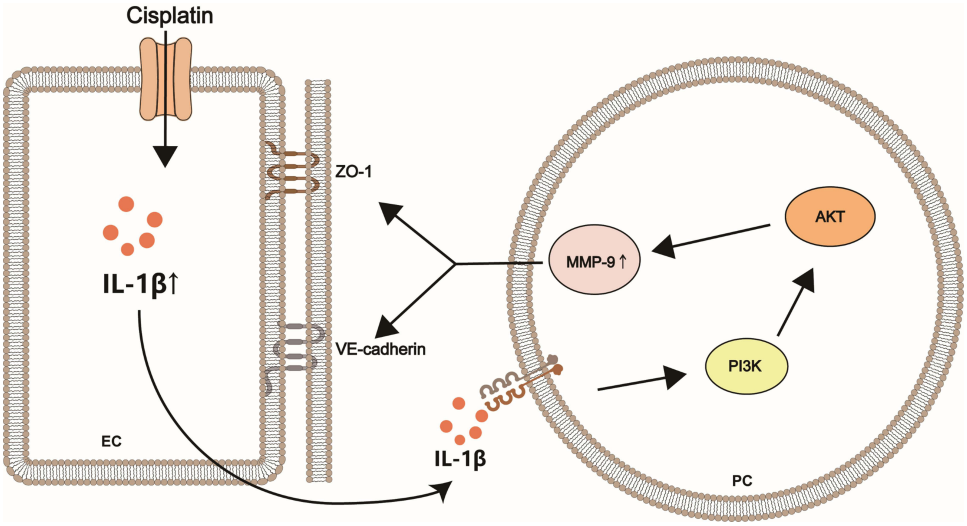
Graphical Abstract



cognitive decline, potentially accelerating cognitive aging, reducing language comprehension and memory capabilities[4,7] and increasing risks of falls and disability.[5] Despite the slow progress in monitoring ototoxicity post-chemotherapy,[8] the increasing incidence of cancer survivors and their extended lifespans exacerbate the issue of CDDP-induced hearing loss.[9] Consequently, investigating CDDP ototoxicity is of paramount importance for geriatric cancer patients, as it holds promise in mitigating hearing loss and safeguarding cognitive and functional abilities.

The severe ototoxicity of CDDP is primarily attributed to its prolonged retention in the cochlea, primarily the stria vascularis (SV).[10] The SV, highly vascularized tissue, maintaining cochlear homeostasis, is guarded by the blood–labyrinth barrier (BLB), akin to the blood–brain barrier (BBB). Disruption of the BLB, a crucial factor in CDDP-induced hearing loss, alters lymphatic fluid electrochemistry, compromising endocochlear potential and elevating hearing thresholds.[11] The BLB, composed of endothelial cells (ECs) interconnected by tight junctions (TJs) and supported by pericytes (PCs),[12,13] not only ensures the stability of the barrier but also regulates angiogenesis and TJs protein expression.[14–17] Our studies indicate that CDDP-induced oxidative stress within the cochlea may disrupt the PCs–ECs interaction,[18] implicating PCs as a potential therapeutic target for mitigating CDDP ototoxicity. Further, research should delve into the BLB's intricate physiological and pathological mechanisms, seeking strategies to protect or restore its functionality. However, research into the specific mechanisms by which CDDP impacts PCs, leading to BLB damage, is still limited.

In recent years, inflammation has emerged as a pivotal mechanism underlying CDDP-induced hearing loss, with cochlear cells exhibiting an initial pathological response that serves as a biomarker for toxicity assessment.[19–22] Clinically, modulating inflammatory processes holds promise for mitigating hearing loss, particularly in light of CDDP's ototoxicity targeting sensory hair cells (HCs), SV, and spiral ganglion neurons, regions vulnerable to inflammatory damage.[19,23] Distinct from pathogen-induced inflammation, CDDP elicits sterile inflammation stemming from tissue injury, prompting inflammatory cells to release mediators like IL-1β, TNF, ROS, NO, and proteases, exacerbating oxidative stress and cochlear damage.[24–26] Tight regulation of these responses is imperative for inner ear preservation. Notably, IL-1β, a key proinflammatory cytokine, activates NF-κB and MAPK pathways, with high expression in the cochlea,[27,28] suggesting a crucial role in regulating cochlear inflammation. While IL-1β upregulation enhances BBB permeability,[29] its potential contribution to CDDP-mediated BLB impairment remains unexplored.

Therefore, we meticulously established a CDDP-induced ototoxicity mice model and a primary cultured in vitro BLB model to validate our research hypothesis, unveil the crucial function and molecular mechanisms of IL-1β in CDDP ototoxicity, identify potential therapeutic targets for preventing and treating CDDP-induced hearing loss.

Materials and Methods

Reagents and Animal

CDDP (P4394), pigment epithelial-derived factor (PEDF) and endothelial Cell Growth Supplements (ECGS) were all purchased from Sigma Corporation in the US; AS101 (IL-1β-inhibitor) was purchased from Selleck Company in the United States; SB-3CT was purchased from MCE Corporation in the United States. Thirty male[30] C57BL/6J mice, aged 6–8 weeks, were sourced from Qingdao Longhe Biotechnology Co., Ltd, the license NO. is SCXK(Lu)2021–0002. These mice were allowed to acclimate for 1 week in temperature (20–25°C) and humidity (50–70%). All animal procedures adhered to the guidelines set forth by the Experimental Animal Ethics Committee at Shihezi University. A total of thirty mice were randomly assigned to three groups (n = 10): Control group, CDDP group and AS101 group. CDDP group received intraperitoneal injections of CDDP (4 mg/kg/d) for 4 days. Control group received the same volume of saline for 4 days. While AS101 group received both CDDP and AS101 (10 μg per mouse) for 4 days.

Auditory Brainstem Response (ABR) Test

Anesthetized mice (n = 6) were implanted with platinum needle electrodes with a diameter of 0.38 mm and a length of 5 cm under the skin of the skull (positive electrode), test ear (negative electrode) and non-test ear (the ground). ABR waveform response is recorded to a series of tone bursts (8/16/24/32 kHz) by gradually decreasing the intensity by 10 dB, starting at 90 dB SPL. The lowest stimulus level at which ABR waves can be observed was determined as the threshold. The changes of threshold and the latency of ABR wave I in each group are observed. Repeat 3 times for each mouse.

Immunohistochemical Analysis and HE Staining

After anesthesia, the cochleas of mice were removed and fixed in 4% paraformaldehyde from anesthetized mouse (n = 6), the cochleas were embedded in paraffin and sectioned to 5 μm slices. Paraffin sections were dewaxed, hydrated, and incubated at 65°C for 2 hours. For HE staining, slices were stained with hematoxylin/eosin and observed under a light microscope. For Immunohistochemical analysis, antigen repair and endogenous peroxidase inhibition were achieved using sodium citrate. Blocking was performed with 10% BSA at 37°C for 1h, followed by washes with PBS. Primary antibodies against ZO-1 (1:200, ab276136), VE-cadherin (1:200, ab205336), IL-1β (1:200, ab283818) were applied overnight at 4°C in a wet box. After repeated washes, secondary antibodies were added and incubated at 37°C for 30 minutes. DAB staining, hematoxylin nuclear staining, acid-alcohol differentiation, and sealing were subsequently performed. Microscopic observation allowed for protein expression analysis.

BLB Leakage Assessment

Four mice per group were used to assess the permeability of the BLB. 2% Evans blue (20 mg/mL/kg) was injected into the tail vein of an anesthetized mouse. After 6 hours of circulation, the bilateral cochlea was quickly harvested and fixed overnight with 4% PFA. The next day, the cochleas were flushed three times with PBS. In order to evaluate the permeability of the cochlear BLB, with care, the SV were carefully separated from the spiral ligament and mounted on slides, and visualized under confocal laser scanning microscope. For each mouse SV, three randomly selected, equal-area fields were assessed to determine the proportion of fluorescent area.

Cell Culture and Identification of PCs and ECs

Both PC and EC cells were isolated from the cochlear SV of 7–12-day-old male C57BL/6J mice. Under aseptic conditions, the incompletely ossified otic capsule wall was pried open with ophthalmic forceps under a dissecting microscope. After complete dissection of the cochlea, the modiolus was exposed. The entire membranous labyrinth was peeled off from the basal turn to the apical turn, allowing isolation of the SV. The SV was thoroughly rinsed three times in sterile D-Hank's

solution and subsequently minced into fragments of approximately 0.15–0.20 mm^3. PC cells were then cultured in an adherent manner in 10% DMEM medium supplemented with PEDF. EC cells were similarly cultured in 10% ECM medium supplemented with ECGS, following the protocols outlined in reference.[5,12] Primary cells were passaged at a fusion degree of 80–90%, and cells from the second to fifth passages were selected for experimental use. To confirm cell identity, fluorescence staining was performed using the pericyte-specific markers Desmin (1:100, ab227651) and PDGFRβ (1:100, ab93534), as well as the endothelial cell-specific markers vWF (1:100, ab11713) and CD31 (1:100, ab7388).

Trans-Epithelial Electrical Resistance (TEER) Value

In vitro BLB model was constructed: separate ECs based on their densities, adding 5×10^4 cells per well to the upper chamber of Transwell for in vitro culture. Allow cells to form a monolayer before adding 3×10^4 PCs suspension per well to the lower chamber, then cultured for 72 hours. For cellular barrier function assessment, Millicell ERS-2 Electrical Resistance System, having undergone rigorous calibration and sterilization procedures, was employed to measure TEER (n = 3). The electrodes were meticulously inserted, with the short end vertically positioned in the upper chamber of the Transwell and the long end in the lower chamber. TER measurements were taken three times per well, and the average values were calculated to ensure accuracy. The specific cellular TEER was determined using the formula: TEER ($\Omega \cdot cm^2$) = (TEERsample - TEERblank) × S (membrane area).

In vitro Barrier Permeability Assessment

Established in vitro BLB model and use FITC extraction as an indicator for evaluating EC permeability in vitro. Detect FITC extran leakage using an ELISA reader by exciting light at 495 nm and emitting at 519 nm (n = 3). Calculate FITC extran permeability over time according to this formula: Permeability (%) = (lower chamber concentration of C) × V (lower chamber)/(upper chamber concentration of C) × V (upper chamber) × 100%. C represents the FITC extraction concentration of the collected liquid and V refers to the volume of the liquid.

Western Blot Analysis

Intervention cells (n = 3) were collected and lysed with RIPA buffer to isolate total cellular protein. Protein concentration was standardized to 20μg/sample using BCA assay. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 2h at RT to minimize non-specific binding. The membranes were then incubated with primary antibodies against ZO-1 (1:1000, ab276136), VE-cadherin (1:1000, ab205336), IL-1β (1:1000, ab283818), MMP9 (1:1000, ab76003), MMP2 (1:1000, ab92536), PI3K (1:1000, CST#4292), p-PI3K (1:1000, CST#17366), p-AKT (1:1000, CST#4060), AKT (1:1000, CST#9272), GAPDH (1:1000, ab8245), and β-actin (1:1000, ab8226) at 4°C overnight. After TBST washing, membranes were incubated with secondary antibodies for specific recognition. Expose the membrane with a chemiluminescence instrument to visualize and quantify protein expression levels using image analysis software.

ELISA Assay

Collect 500μL blood samples from the mouse's inner canthus vein (n = 6) or cell supernatants (n = 3) and centrifuge at 300g/min for 5 minutes to isolate serum or cell supernatant. Utilizing the Lianke Biological ELISA Kit and Sanyo's multi-functional fluorescent enzyme-linked immunosorbent assay (Japan), measure the concentrations of serum IL-1β and IL-1β in cell supernatants. Strictly adhere to the standard reference provided by the kit manufacturer.

Statistics Analysis

Data were replicated a minimum of three times per group, and all experimental outcomes are presented as mean ± SD. Statistical analysis was performed using SPSS 25.0 software. A comparison between the two groups was conducted using the Student's *t*-test, while one-way analysis of variance (ANOVA) was employed for comparing multiple groups. Set $P < 0.05$ as the threshold for statistically significant differences.

Results

IL-1β Contributes to Hearing Loss and SV Damage Induced by CDDP

Given the crucial role of the inflammatory mediator IL-1β in triggering inflammatory gene cascades and modulating functions in injured tissues, we aim to investigate whether cochlear IL-1β contributes to CDDP-induced ototoxicity. So we designed an experimental protocol as outlined in Figure 1A. Mice received a four-day treatment of CDDP (4 mg/kg/d), saline, or AS101 (an IL-1β inhibitor). Immunohistochemical results for IL-1 β are reported in Figure 1B. IL-1β protein expression in the SV of the cochlea was weak in normal mice. Notably, the CDDP group exhibited a significant increase in IL-1β staining score compared to the control. Administration of the IL-1β inhibitor AS101 conspicuously mitigated this enhancement, indicating its suppressive effect on IL-1β expression (Figure 1C). Furthermore, similar changes were detected in the serum of CDDP-injected mice (Figure 1D). CDDP treatment significantly increased IL-1β level compared to controls, which was notably suppressed by AS101.

To further elucidate the role of the inflammatory cytokine IL-1β in CDDP-induced hearing loss, ABR testing was conducted before and after treatment to assess changes in hearing thresholds. As shown in Figure 1E, the control mice exhibited normal hearing across various frequencies (8 kHz, 16 kHz, 24 kHz, 32 kHz) with thresholds around 20 dB in 32kHz (Figure 1E), while CDDP-treated mice displayed significantly elevated ABR thresholds to around 75 dB in 32kHz, with a frequency-dependent increase in hearing loss. Notably, mice treated with AS101 exhibited reduced ABR thresholds and partial improvement in hearing loss (Figure 1E and F).

Consistent with our findings, as shown in Figure 1G, HE staining results further supported the role of IL-1β in CDDP-induced ototoxicity. In control mice, the SV of the cochlea exhibited a normal morphology with clear structure. However, CDDP treatment resulted in atrophy of the SV, structural disruption, and increased vacuolation. Notably, AS101 intervention partially ameliorated the atrophy and reduced vacuolation. These results suggest that AS101 can attenuate CDDP-induced hearing loss and atrophy of the SV in mice, providing further evidence for the involvement of IL-1β in CDDP ototoxicity.

CDDP Increases the Permeability of BLB Through IL-1β in Mouse Cochlear SV

To further probe the role of IL-1β in CDDP-induced changes in the permeability of BLB, Evans blue dye was injected into mice via the tail vein. This dye binds to plasma proteins, generating a red fluorescence visible under fluorescence microscopy, enabling us to assess Evans blue leakage into the SV. Red fluorescence was confined to the microvasculature in normal mice, with minimal dye leakage. However, in CDDP group, red fluorescence was evident outside the microvasculature (Figure 2A). Notably, treatment with AS101, an IL-1β inhibitor, attenuated the fluorescence intensity and reduced Evans blue leakage (Figure 2A).

Given the crucial role of ZO-1 and VE-cadherin in maintaining the integrity and function of endothelial cell tight junctions, we also examined the expression of these proteins. Immunohistochemical analysis demonstrated that ZO-1 and VE-cadherin proteins are primarily expressed in the SV of the cochlea, accompanied by high staining intensity (Figure 2B). Compared to the control group, CDDP treatment significantly reduced the staining intensity of both ZO-1 and VE-cadherin proteins in SV. Interestingly, AS101, intervention partially reversed the CDDP-induced downregulation of these proteins. Further statistical analysis demonstrated that AS101 significantly ameliorated CDDP-induced Evans blue leakage (Figure 2C) and the changes in ZO-1 and VE-cadherin protein staining score (Figure 2D and E).

Collectively, these findings suggest that CDDP, via IL-1β, may downregulate ZO-1 and VE-cadherin expression in the SV, leading to increased BLB permeability and potentially compromising cochlear function. These insights into CDDP-induced ototoxicity may pave the way for novel therapeutic strategies to mitigate hearing loss.

CDDP Induced the Secretion of IL-1β from ECs of SV, Rather Than PCs

To delve deeper into the origins of IL-1β in SV, we initially focused on the isolation, primary cultivation, and identification of ECs and PCs. Examination of Figure 3A revealed that the primarily cultured ECs exhibited a flat, cobblestone-like morphology and positively expressed the endothelial markers vWF and CD31 while remaining negative

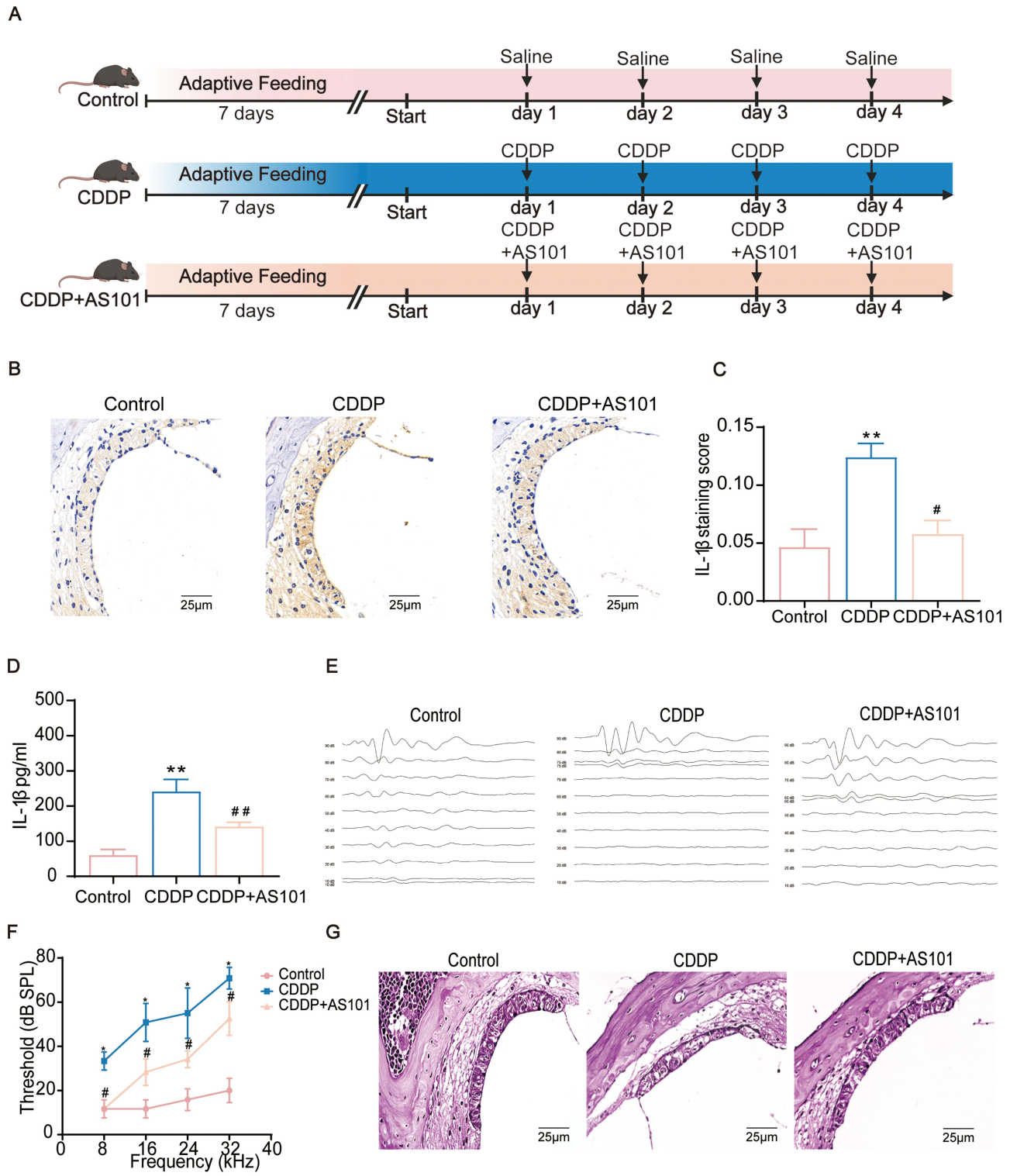


Figure 1 Effects of IL-1β on CDDP-induced ototoxicity in mouse. (**A**) Schematic diagram of animal experiment in male C57BL/6 mouse (drugs administration protocol). (**B**) Immunohistochemical staining image of IL-1 β in cochlear SV from various groups. (**C**) Quantitation of IL-1 β stainning score in cochlear SV (n=6); (**D**) Quantitation of serum IL-1 β level in various group of mice by ELISA (n = 6). (**E**) Representative ABR waveforms in various groups. (**F**) Change of ABR hearing thresholds in various groups (n=6). (**G**) Morphological changes of cochlear SV in various groups. Scale bar = 25 μm. $^{*}P < 0.05$, $^{**}P < 0.001$ vs Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs CDDP.

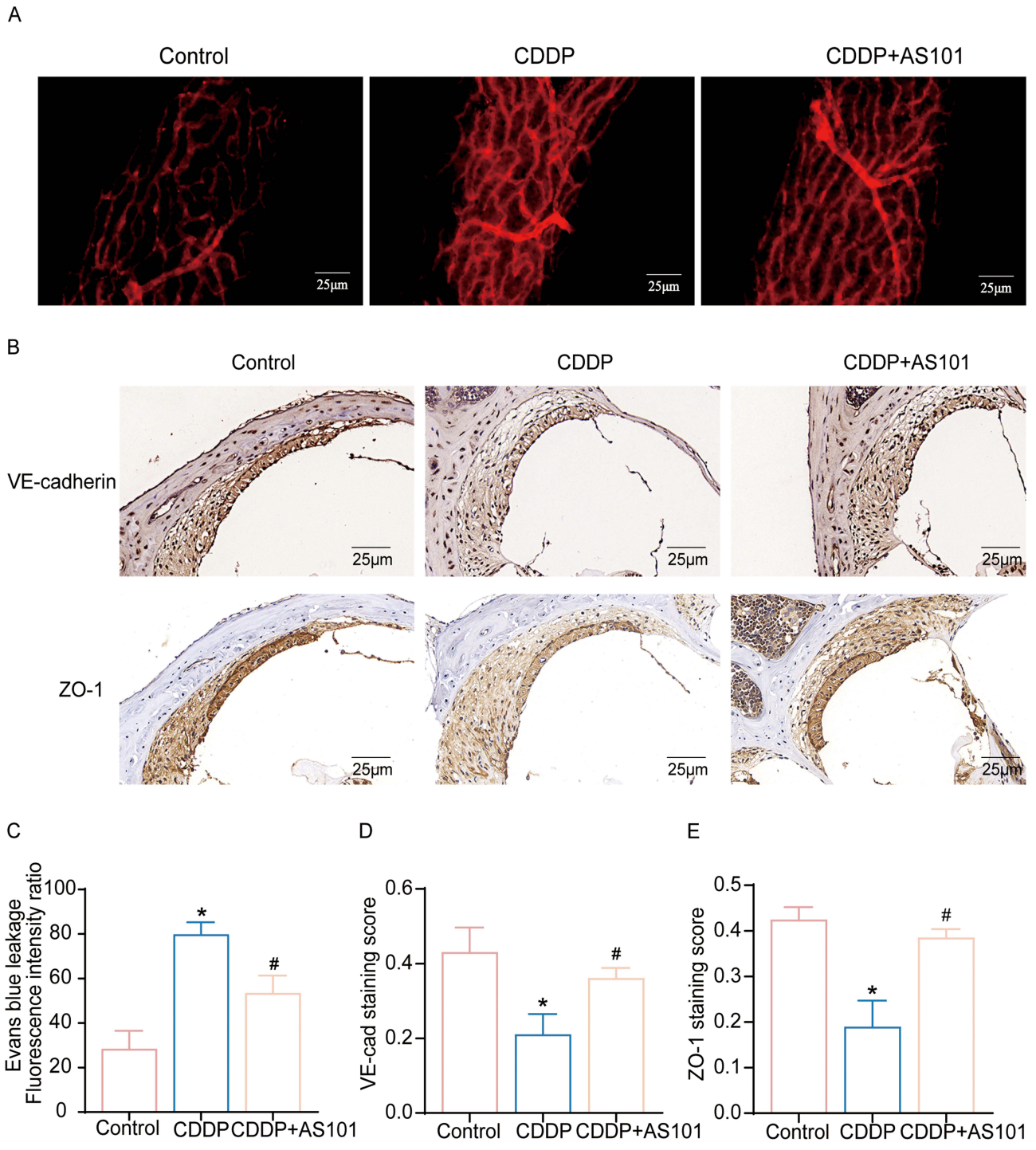


Figure 2 Effect of IL-1β on the permeability of BLB. (**A**) Images of Evans blue leakage of SV in various groups (Scale bar = 25 μm). (**B**) Immunohistochemical staining of ZO-1 and VE-cadherin proteins in the cochlear SV (Scale bar = 25 μm). (**C**) Quantitation of leakage fluorescence density of SV in various groups (n=3). (**D** and **E**) Quantitation of VE-cadherin and ZO-1 protein expression in various group of mice (n=3). *$P < 0.05$ vs Control, #$P < 0.05$ vs CDDP.

for the PC marker Desmin. Meanwhile, the PCs appeared larger in size, adopting a long spindle or polygonal shape, and positively expressed Desmin and PDGFR β, but did not express the ECs marker vWF (Figure 3A).

Subsequently, we investigated the effects of CDDP on IL-1β secretion in ECs and PCs. Various concentrations of CDDP (5, 10, 15, 20, and 25 μM) were applied to ECs and PCs for 12 hours, followed by quantification of IL-1β levels in the culture supernatants using ELISA. As illustrated in Figure 3B, when compared to the control group, CDDP induced a dose-dependent

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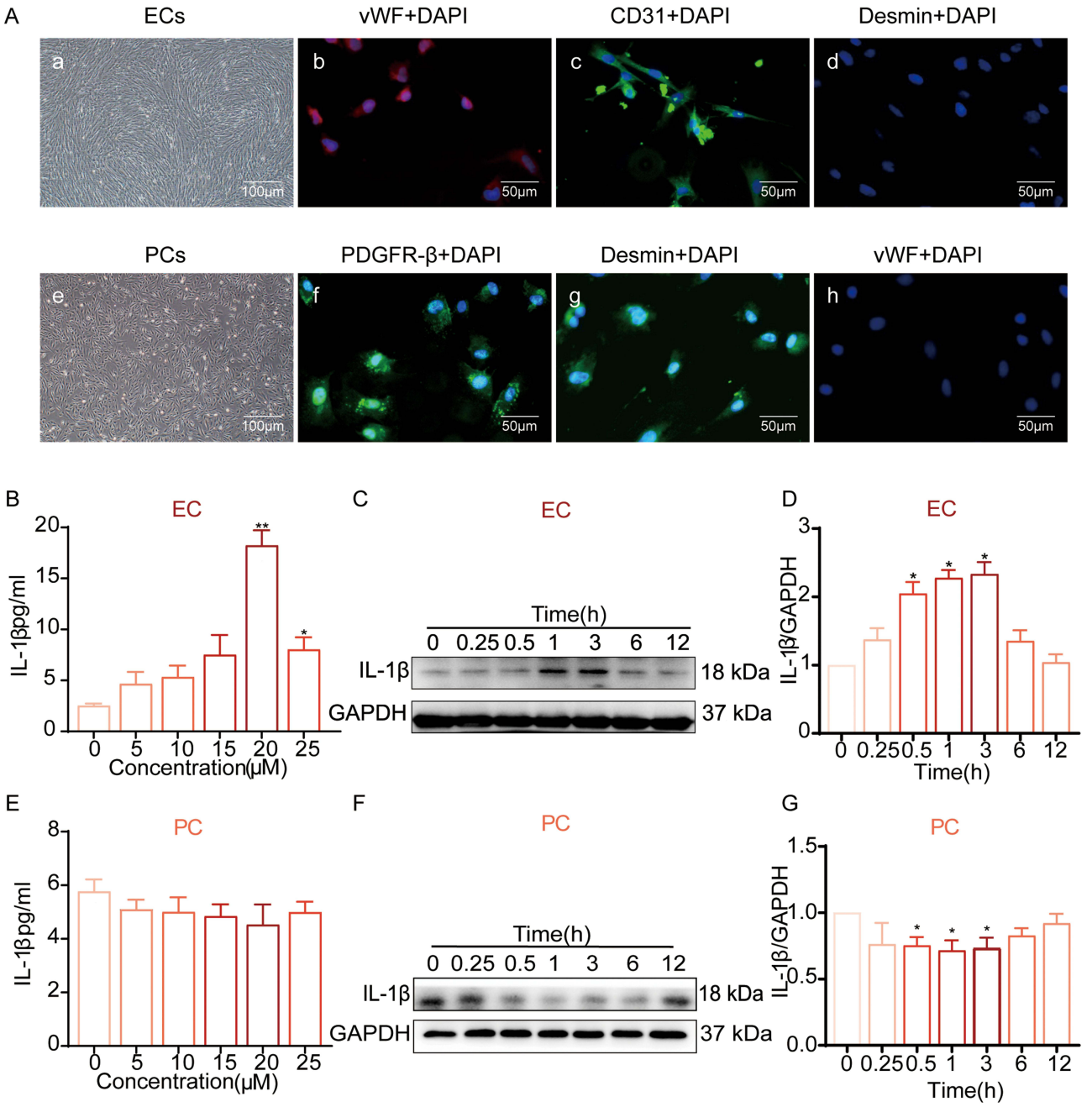

Figure 3 Change of IL-1 β level in ECs and PCs after CDDP treatment in vitro. (**A**) Immunofluorescence labeled primary cultured PCs and ECs. PCs are labeled with PDGFR (green), Desmin (green); ECs are labeled with vWF (red), CD31 (green); DAPI (blue) Scale bar=100, 50μm. (**B**) IL-1β level from ECs after various dose of CDDP treatment by ELISA (n =3) (**C**) IL-1β secretion by ECs after various intervention time of CDDP by Western blot. (**D**) Quantitation of IL-1β secretion by ECs (n =3). (**E**) IL-1β level from PCs after various dose of CDDP treatment by ELISA (n =3). (**F**) IL-1β secretion by PCs after various intervention time of CDDP by Western blot. (**G**) Quantitation of IL-1β secretion by PCs (n =3).* $P< 0.05$, ** $P< 0.01$ vs Control.

increase in IL-1β secretion from ECs, with the most pronounced effect observed at a concentration of 20 μM ($P < 0.01$). Consequently, a concentration of 20 μM CDDP was selected for subsequent experiments. To further analyze the temporal effects of CDDP treatment on IL-1β protein expression in ECs and PCs, we conducted a time-course experiment with six designated time points (0, 0.25, 0.5, 1, 3, 6, and 12 hours) during a 12-hour exposure to 20 μM CDDP. Western blot analysis was used to assess protein levels of IL-1β. Our findings revealed that compared to the control group (0 h group), the expression level of IL-1β in ECs was significantly increased in the 12-hour CDDP treatment, peaking at 3-hour time point in ECs (Figure 3C). These differences are statistically significant (Figure 3D). In contrast, under identical CDDP treatment conditions,

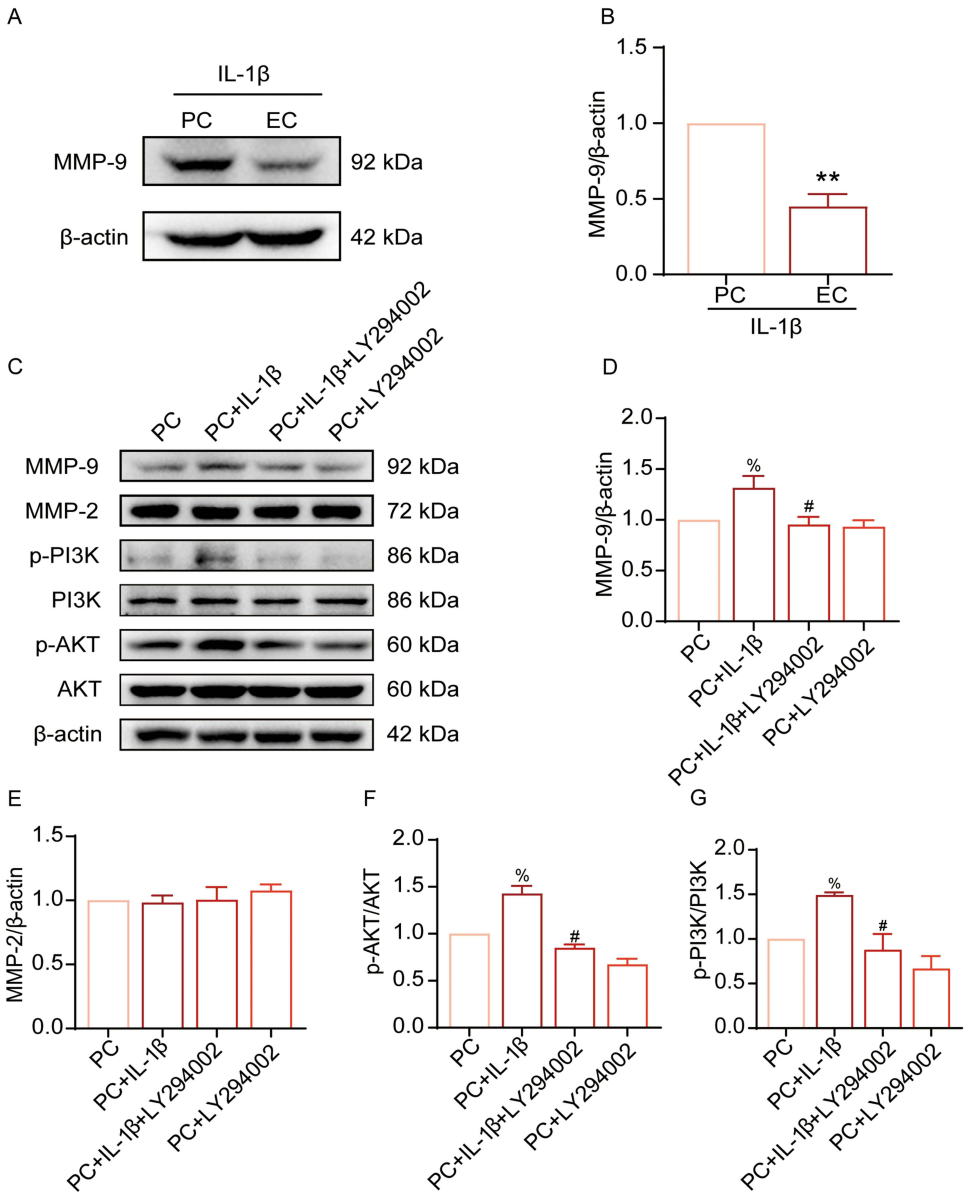

Figure 4 IL-1β regulates MMP9 through PI3K/Akt signaling pathway in vitro. (**A**) Effects of IL-1β on the expression of MMP9 on PCs and ECs via Western blot. (**B**) Quantitation of MMP9 expression in various group (n=5), $^{**}P< 0.01$ vs PC+ IL-1β. (**C**) Western blot detection of MMP9, MMP2, p-PI3K, PI3K, p-AKT, and AKT protein expression changes in each group. (**D**–**G**) Quantitative analysis of MMP9, MMP2, P-AKT/AKT and P-PI3K/PI3K expression in different groups (n=3), $^{\%}P<0.05$ vs PC, $^{\#}P<0.05$ vs PC+IL-1β.

examination of PCs revealed no change in IL-1β levels (Figure 3E). Notably, subsequent Western blot analysis of the temporal effects of CDDP on PCs showed a progressive decline in IL-1β expression over time, reaching the lowest point occurring at 1 hour (Figure 3F and G). These results implicated a role for EC-derived IL-1β in CDDP-induced SV inflammation.

IL-1β Upregulates MMP9 in PCs via PI3K/AKT Pathway

Previous reports have shown that inflammatory responses can stimulate the overexpression of MMP9 in pericytes, which plays a crucial role in blood–brain barrier (BBB) injury.[31] Accordingly, we conducted in vitro studies to elucidate the molecular mechanisms underlying the regulation of MMP9 secretion by IL-1β. After IL-1β treatment, we observed significantly higher MMP9 protein expression in PCs compared to ECs within the co-culture system (Figure 4A), which

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showed statistically significant differences ($P < 0.01$) (Figure 4B). This suggests that the increased MMP9 expression is primarily due to the action of IL-1β on PCs rather than ECs in vitro.

Given the established role of the PI3K/AKT pathway in MMP9 regulation,[32,33] we employed the specific PI3K/AKT pathway inhibitor LY294002 to intervene and observed changes in MMP9 secretion by PCs. Western blot analysis demonstrated a notable increase in MMP9 protein expression in PC+IL-1β group compared to the PC group. Notably, the addition of LY294002 led to a significant reduction in MMP9 expression compared to the PC+IL-1β group (Figure 4C and D). In contrast, MMP2 protein expression remained stable and unaffected in all groups. (Figure 4C and E). Concurrently, the ratios of P-PI3K/PI3K and P-AKT/AKT were also markedly elevated in this group compared to the PC group. After LY294002 intrevention, the changes in the ratios of P-PI3K/PI3K and P-AKT/AKT were consistent with the aforementioned trend in MMP9 protein expression (Figure 4C, F and G). These findings collectively suggest that IL-1β may potentiate MMP9 expression in PCs via activating the PI3K/AKT signaling pathway, ultimately leading to enhanced BLB permeability.

IL-1β Damages the Endothelial Barrier Permeability by Regulating MMP9

To clarify whether IL-1β influences the function of BLB permeability through MMP9, we established a BLB model using ECs-PCs co-culture in vitro (Figure 5A). We further investigated the endothelial barrier permeability using TEER values and FITC-dextran fluorescence permeability as indicators. As shown in Figure 5B, EC+PC co-culture group exhibited higher TEER values than the EC group. However, unexpectedly, TEER values in EC+IL-1β group were significantly lower in contrast to the EC group ($P < 0.05$). When compared to EC+PC group, TEER values in the EC+PC+IL-1β group were significantly reduced. Notably, pretreatment with MMP9 inhibitor SB-3CT ameliorated the IL-1β-induced decrease in TEER values in the co-culture group. Similarly, EC+PC group showed lower FITC-dextran fluorescence permeability compared to EC group, while EC+IL-1β group exhibited increased permeability (Figure 5C). When compared to EC+PC group, the fluorescence permeability in EC+PC+IL-1β group was significantly elevated. SB-3CT pretreatment attenuated these IL-1β-induced changes (Figure 5C). This further supports the notion that IL-1β increases the permeability of the BLB by regulating MMP9. We further delved into the influence of MMP9 in the IL-1β on TJ-associated proteins, specifically ZO-1 and VE-cadherin. Our findings revealed that, compared with EC group, ZO-1 expression was markedly upregulated in EC+PC group, but significantly downregulated in EC+IL-1β group (Figure 5D and E). Notably, EC+PC +IL-1β group exhibited a notable decrease in ZO-1 protein expression compared to EC+PC group, and pretreatment with SB-3CT effectively attenuated these IL-1β-induced changes (Figure 5D and E). Similarly, VE-cadherin expression followed a comparable pattern to ZO-1 (Figure 5D and F).

Taken together, our results suggest that co-culture mode is helpful to increase the stability of the endothelial barrier, and EC-produced IL-1β may enhance BLB permeability by modulating MMP9 expression in PCs.

Discussion

A prior study hinted at inflammation as the initial pathological change underlying CDDP-induced hearing loss,[19,22] yet the precise mechanisms underlying this process remain elusive. In this study, we observed a decline in hearing capacity and an elevation in the BLB permeability of the SV in C57BL/6J mice exposed to CDDP. Further cellular studies revealed that CDDP could provoke the release of IL-1β from ECs, triggering the activation of the PI3K/AKT signaling pathway, then led to an upregulation of MMP9 expression in pericytes, enhancing BLB permeability and contributing to the onset of hearing loss.

Consistent with prior reports by Tejbeer Kaur[34] and Sheth,[35] our study corroborates that CDDP can indeed cause hearing loss in mice. Besides, SV atrophy, a pathological feature observed in multiple inner ear disease models,[4] is also evident in our experiments. Notably, CDDP not only elevates the hearing threshold but also significantly alters the morphology of the SV, characterized by atrophy and increased vacuolation. Previous investigations have linked BLB permeability in the SV with hearing function, and our BLB leakage assessment by Evans blue in mice further strengthen this hypothesis, indicating that an increase in BLB permeability is a pivotal mechanism underlying CDDP-induced ototoxicity, providing insights into its pathophysiological processes.

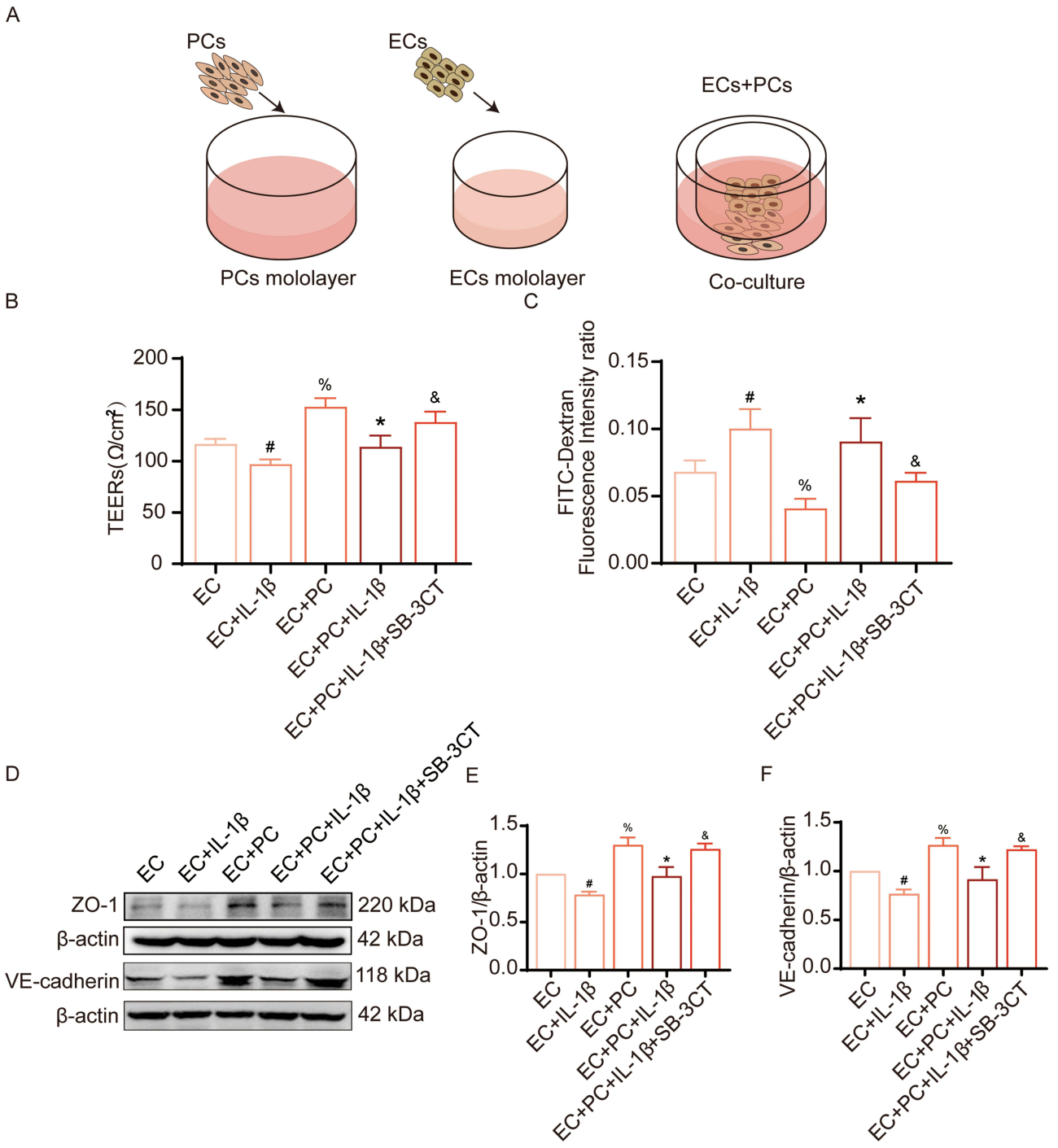

Figure 5 Effect of MMP9 on IL-1β induced endothelial barrier permeability. (**A**) Schematic diagram of constructing a BLB model in vitro. (**B** and **C**) TEER values and FITC-dextran fluorescence in different groups (n=4). (**D**) Expression of ZO-1 and VE-cadherin protein in ECs by Western blot in various group. (**E** and **F**) Quantitation of ZO-1 and VE-cadherin protein expression in various group (n=3). $^{\#}P<0.05$, $^{\%}P<0.05$ vs EC, $^{*}P<0.05$ vs EC+PC, $^{\&}P<0.05$ vs EC+PC+IL-1β.

Recent research has shown that inflammation can lead to an increase in the BLB permeability.[36,37] Qin et al[38] observed that abnormally high levels of IL-1β in the SV contribute to increased BLB permeability. Inflammation often leads to the elevation of inflammatory cytokines, among which IL-1β stands out as a potent proinflammatory cytokine.[31] This cytokine plays crucial roles in various physiological and pathological processes, including cell proliferation, differentiation, apoptosis, inflammation induction, and immune response regulation.[39] Our results reveal that CDDP triggers an enhanced inflammatory response in the SV, characterized by the upregulation of IL-1β expression.

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Intervention with the IL-1β inhibitor AS101 not only partially reverses CDDP-induced hearing loss but also mitigates the adverse effects of CDDP on BLB permeability.

To delve deeper into the role of IL-1β in CDDP-induced BLB damage, we primarily cultured of ECs and PCs derived from the cochlear SV and established in vitro BLB model. In individual culture systems, our observations revealed that CDDP stimulated IL-1β secretion from ECs rather than PCs. Similarly, when intervention was applied to the co-culture model of ECs and PCs, it resulted in augmented IL-1β secretion. This indicates that ECs are the primary source of CDDP-induced IL-1β upregulation, while PCs may mitigate inflammation by inhibiting the NFAT pathway.[40]

MMPs, zinc-dependent endopeptidases, play a crucial role in maintaining inner ear homeostasis. MMP2 and MMP9, members of the MMP family, are capable of degrading collagen IV, a major component of ECM, thereby closely linked to the regulation of BBB /BLB permeability.[32,41] Notably, Leukemia cells secrete MMP2 and MMP9 to degrade tight junction proteins ZO-1, Occludin, and Claudin-5, disrupting the BBB and facilitating invasion into the central nervous system.[33] Furthermore, aminoglycoside intervention or noise exposure significantly upregulates cochlear MMPs expression, while MMP inhibitors alleviate cochlear hair cell apoptosis,[32,42,43] These findings indicated the necessity of maintaining MMPs activity for normal cochlear function. Our experiments revealed that inflammatory cytokine IL-1β upregulated MMP9 expression in PCs, with MMP2 expression remaining unchanged. Notably, MMP9 expression was higher in PCs than in ECs. This observation aligns with previous studies demonstrating that the stimulatory effect of IL-1β on MMP9 protein expression in PCs of the BBB, which is a key factor contributing to increased barrier permeability.[44] These discoveries further underscore the critical role of PCs as the primary source of MMP9 within the cochlea, playing a pivotal role in modulating the BLB permeability. Additionally, our results demonstrate that IL-1β intervention significantly reduced ZO-1 and VE-cadherin expression in ECs, which was ameliorated by MMP9 inhibitors. This suggests that IL-1β-induced MMP-9 secretion from PCs and subsequent disruption of tight junction proteins expression, which contribute significantly to increased BLB permeability. However, our understanding of PCs mechanisms remains limited. The role of PCs in maintaining BLB integrity is complex, and extends beyond the regulation mediated by IL-1β-induced MMP9. Indeed, PCs exhibit a multifaceted functionality that encompasses a wide array of molecular interactions and signaling cascades. For instance, PCs have been implicated in the regulation of vascular tone and blood flow within the cochlear microenvironment, processes that are crucial for auditory function. They achieve this through the release of vasoactive substances and the modulation of vasoconstrictor and vasodilator responses.[45] Furthermore, PCs are known to interact with endothelial cells, neurons, and fibroblasts, forming a complex network of cellular communications that is essential for maintaining cochlear homeostasis.[14] Therefore, a comprehensive analysis of PCs function and interactions other cell types within the cochlear microenvironment is necessary.

The PI3K/Akt pathway serves as a critical regulator of various physiological and pathological states, encompassing cell proliferation, migration, apoptosis, and neovascularization.[46] Accumulating evidence suggests a crucial role of this pathway in MMPs-induced damage. Activation of this pathway upregulates MMP9 expression in hepatocellular and renal cell carcinoma,[47,48] facilitating tumor cell invasion and metastasis. Consistent with this, our Western blot analysis confirmed the involvement of the PI3K/Akt pathway in IL-1β-mediated regulation of MMP9 secretion in PCs, thereby altering the BLB permeability. These findings have important implications for human clinical research. By identifying the inflammation-mediated PI3K/AKT activation and MMP9 upregulation as key mechanisms in CDDP-induced hearing loss, our study provides potential targets for therapeutic intervention. Moreover, given the pivotal role of the PI3K/AKT pathway in IL-1β-mediated MMP9 upregulation, we intend to screen for small molecule inhibitors of this pathway that could potentially block the deleterious effects of IL-1β on the BLB. Such inhibitors could represent a novel class of otoprotective agents.

Moreover, the PI3K/Akt pathway's significance played a crucial role in cochlear hair cell survival. AKT regulates hair cell survival and protects them from ototoxic drugs like gentamicin.[49] Recent studies suggest that the PI3K-AKT signaling can serve as a therapeutic target for harmful stimuli (eg, gentamicin and CDDP) and a crucial factor for cell survival.[50,51] In noise-induced cochlear injury, deferoxamine successfully induces mesenchymal stem cell homing via the PI3K-AKT pathway.[52] To comprehensively understand the complex functions of the PI3K/Akt pathway in the auditory system, further research is required to unravel its detailed mechanisms.

Furthermore, future research can employ transcriptome analysis to identify upstream differential genes of inflammatory factors, potentially uncovering novel pathways and targets for mitigating CDDP-induced hearing loss in cancer

patients. Addressing these limitations is vital for advancing our understanding of CDDP ototoxicity and translating findings into clinical practice.

Conclusions

In summary, this study focused on IL-1β's role in CDDP-induced hearing loss. Meticulous experiments confirmed the crucial involvement of IL-1β in CDDP-induced hearing impairment. Further investigations revealed that IL-1β modulates the PI3K-AKT signaling pathway, particularly by increasing the production of MMP9 in pericytes, thereby increasing BLB permeability. This offers a new perspective on CDDP ototoxicity and suggests future clinical directions for safer, more effective cancer treatments. However, current research on the mechanisms of CDDP ototoxicity is limited, particularly regarding its molecular effects on ECs inflammatory factors. Therefore, transcriptome analysis can be employed to screen for upstream differential genes of inflammatory factors, further elucidating CDDP's ototoxicity mechanisms.

Data Sharing Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to reasons of privacy.

Ethical Approval

All animal procedures adhered to the guidelines set forth by the Experimental Animal Ethics Committee at Shihezi University. Ethical approval for all experimental procedures was obtained from the Committee of Animal Experimental Ethics of The First Affiliated Hospital of Medical College, Shihezi University (permit NO. is A2022-167-01).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare no conflict of interest.

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