

Original Article

Cisplatin-induced stria vascularis cell apoptosis by increased activation of p66Shc

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Abstract: *Objective:* To explore the role of the p66Shc in the Cisplatin induced apoptosis of stria vascularis cells in the cochlea. *Methods:* Thirty healthy male Kunming mice (weight 20-25 g; 6-8-week-old) were administered a daily intraperitoneal injection of cisplatin (low-dose group: 3 mg/kg, high-dose group: 6 mg/kg) and an equivalent volume of normal saline for the control group in the abdomen for 7 days. Auditory brainstem response thresholds (ABRs) were measured before and after cisplatin administration. TUNEL assays were used to test the apoptotic cells. The location of p66Shc expression in the cochlea was evaluated by immunofluorescence. Western blot analyzed the whole protein levels of p66Shc and pSer36-P66Shc in the cochlea. *Results:* The p66Shc protein was only expressed in the stria vascularis cells of the cochlea. After cisplatin treatment, ABR thresholds in the mice increased, TUNEL-positive apoptosis cells were found in the stria vascularis cells, and whole protein levels of p66Shc and pSer36-P66Shc were increased significantly in the stria vascularis cells. *Conclusion:* The increase and activation of p66Shc may play a critical role in Cisplatin-induced stria vascularis cells apoptosis.

Keywords: Cisplatin, ototoxicity, stria vascularis, p66Shc

Introduction

Cisplatin (cis-diamine dichloroplatinum II; CD-DP) is a highly effective chemotherapeutic agent that is widely used to treat a variety of soft tissue neoplasms, including ovarian, testicular, cervical, head and neck, lung, and bladder cancer [1]. However, CDDP therapy is limited due to cellular resistance and severe side-effects such as nephrotoxicity, neurotoxicity, and ototoxicity in the normal tissues [2]. Some auditory studies reported that 75-100% of the patients treated with CDDP developed a bilateral, symmetric, irreversible, high-frequency hearing loss as the dose or the number of treatments increased [3]. Some studies have stated that production of reactive oxygen species (ROS) could cause cellular stress and activate the apoptotic pathway that leads to cell death associated with CDDP-induced ototoxicity [4]. Although ROS are considered as key factors in CDDP-induced ototoxicity, the underlying mechanism is poorly understood.

p66Shc protein is a Src homolog and collagen homolog (Shc) adaptor protein, encoded by the

mammalian *Shc A* gene [5]. In addition, it is known to play a crucial role in the regulation of intracellular ROS levels and upregulation during apoptosis [6, 7]. Previous studies reported that CDDP activates the adaptor protein, p66Shc, by phosphorylating its Ser36 residue in renal proximal tubule cells, and it is vital for CDDP-induced nephrotoxicity [8, 9]. The Ser36 residue phosphorylated p66Shc (pSer36-p66Shc) protein is translocated to the mitochondrial intermembrane space, consequently inducing the production of ROS by oxidized cytochrome c (cyt C) [10]. p66Shc protein is also expressed in the lateral wall of the cochlea and associated with the D-gal-induced presbycusis [11]. However, no study of p66Shc in CDDP-induced ototoxicity has yet been reported.

Several studies demonstrated that cisplatin-induced ototoxicity is predominantly responsible for the sensory hair cell death in the cochlea of the peripheral auditory sensory system [12]. However, cisplatin-induced auditory dysfunction is composed of multi-level effects that might damage the stria vascularis (SV) cells in the lateral wall of the cochlea, thereby resulting

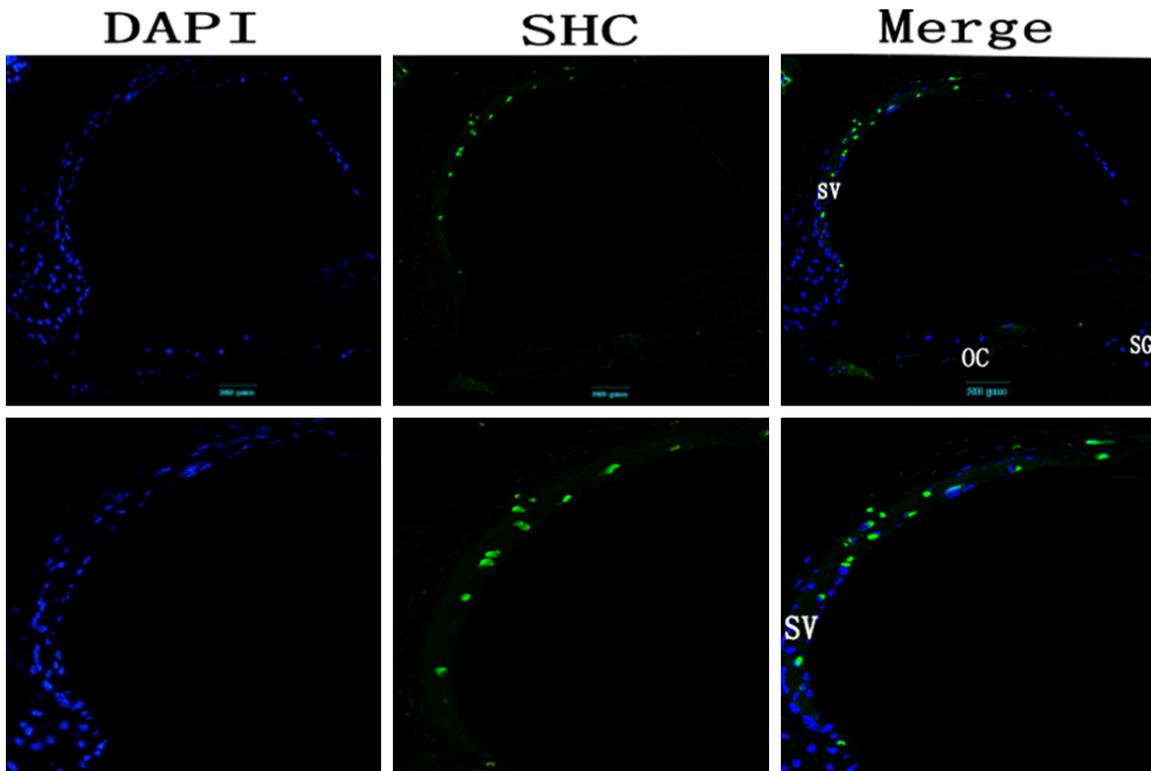


Figure 1. Immunostaining for p66Shc shows expression of p66Shc as identified by green fluorescence; localized only in the cytosolic part of the SV cells, none in the OC cells and SG cells. Bar represents 20 μ m. SV: stria vascularis, OC: organ of Corti, SG: spiral ganglion.

in the loss of endolymphatic potential (EP) or breakdown of the blood labyrinth barrier (BLB) [13]. Thus, we focused on the SV in the current study and established a CDDP-induced ototoxicity mouse model to investigate the total levels of p66Shc and the pSer36-p66Shc in the SV cells after CDDP-treatment. Our results suggest that the p66Shc and pSer36-p66Shc proteins might play a critical role in CDDP-induced SV cells apoptosis.

Materials and methods

Reagents

Cisplatin (Qilu Pharmaceutical Co., China), ketamine (Gutian Fujian Pharmaceutical Co., China), chlorpromazine (Shanghai He Feng Pharmaceutical Co., China), paraformaldehyde fixation fluid, Total Protein Isolation Kit, BCA Protein Assay Kit, SDS-PAGE Kit, ECL Kit (Nanjing KeyGen Biotech. Co., China), TUNEL Kit (Roche, USA), anti-HC rabbit polyclonal antibody, anti-Ser36-P-p66Shc antibody (Abcam, USA), horseradish peroxidase (HRP)-conjugated

goat anti-rabbit and goat anti-mouse antibodies (Santa Cruz, USA) were used in this study.

Animal and treatment

Thirty healthy male Kunming mice (weight 20-25 g; 6-8-week-old) with sensitive Preyer's reflex were purchased from the Comparative Medical Center of Yangzhou University. The animals were maintained at the Animal Experimental Center of Nanjing Medical University and allowed free access to water and diet. The animals were randomly assigned to three groups (ten mice in each group) as follows: a low-dose (LD) group, high-dose (HD) group, and control group. The mice were treated daily with an intraperitoneal injection of CDDP (LD group 3 mg/kg CDDP, HD group 6 mg/kg CDDP) and an equal volume of normal saline (NS) in the control group via abdomen for 7 days. All efforts were made to minimize the suffering of the animals and reduce their number. All procedures were performed in compliance with the Laboratory of Animal Care and Use Committee of the Nanjing Medical University.

Table 1. The ABR threshold (dB SPL)

Group	Mean \pm SD	P-value
NS	36.00 \pm 5.16	
LD	41.50 \pm 5.80	0.105 (compared to NS)
HD	58.00 \pm 10.06	< 0.001 (compared to NS and LD)

A significant increase is observed in the ABR threshold in the HD group as compared to the NS group, P -value < 0.001. NS: the group treated with normal saline; LD: the group treated with 3 mg/kg cisplatin; HD: the group treated with 6 mg/kg cisplatin. ABR: auditory brainstem-evoked responses.

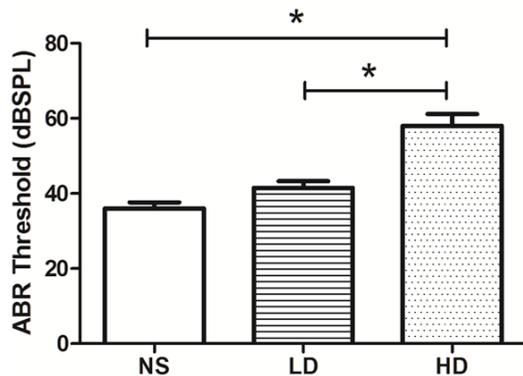


Figure 2. The ABR thresholds of the HD group were significantly higher than the NS and LD groups. NS: the group treated with normal saline; LD: the group treated with 3 mg/kg cisplatin; HD: the group treated with 6 mg/kg cisplatin. *, $p < 0.05$.

Auditory brainstem response thresholds (ABRs)

At the beginning and end of the treatment (pre-test and post-test), the hearing functions of the mice were assessed by ABR. The click-evoked ABRs were measured using a signal processor (7S11; NEC Sanei Instrument, Tokyo, Japan) under anesthesia using a mixture of ketamine (120 mg/kg) and chlorpromazine (20 mg/kg). The sound delivery tube of the insert earphone was tightly fit into the external auditory canal. Subdermal electrodes were placed over the vertex (active), right mastoid (reference), and left mastoid (ground). Tone bursts of pure tones from 6 to 32 kHz (0.1 ms rise/fall time, 10 ms total duration, 11/s repetition rate) were presented monaurally. Responses were filtered (0.15-3 kHz), digitized, and averaged (across 250 discrete samples at each frequency-level combination). Thresholds were estimated to occur between the lowest stimulus level, wherein an ABR response in wave V was observed, and the next lowest level without response. All evaluations were carried out by an observer blinded to the experimental conditions.

Tissue sample preparation

All the animals were executed immediately following post-treatment ABR under deep anesthesia with a mixture of ketamine (120 mg/kg) and chlorpromazine (20 mg/kg). The cochleae from the animals were collected. For terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) and immunofluorescence experiments, the right cochlea of each animal was perfused slowly with 4% paraformaldehyde in phosphate-buffered saline (PBS) and maintained in the fixative at 4°C overnight. Subsequently, the cochleae were decalcified with disodium EDTA for 1 week. These decalcified cochleae were then paraffin-embedded and 5-mm sections were sliced from the block, and mounted on glass slides. For DNA and protein detection, the lateral wall of the left cochlea of each mouse that included the SV and spiral ligament was isolated under a stereomicroscope (Zeiss, Germany). The total proteins were isolated using the Total Protein Isolation Kit and concentrations estimated by the BCA Protein Assay Kit; BSA was used as the standard.

Immunohistochemical studies

After deparaffinization, rehydration, antigen retrieval, and nonspecific antigen site blocking, the primary antibody to SHC (1:50) was incubated on the sections overnight at 4°C, followed by 1 hour incubation with the fluorescently tagged secondary antibody. Concurrently, control staining was performed without the primary antibody. Images were captured with a laser scanning confocal microscope (Nikon, Japan).

TUNEL assay

Apoptotic cells were detected *in situ* utilizing the TUNEL assay. The TUNEL Kit (Roche, USA) was used and all the procedures were carried out according to the manufacturer's instructions. The optical microscope (Olympus, Provis) was used to capture the images with a $\times 400$ objective. Brown particles could be found in the nucleus of the positive cells.

Western blot

Total protein level of p66Shc, p52Shc, and p46Shc were detected using an antibody against SHC, which detects the three isoforms simultaneously by binding to a similar domain in all the three proteins. The total protein level

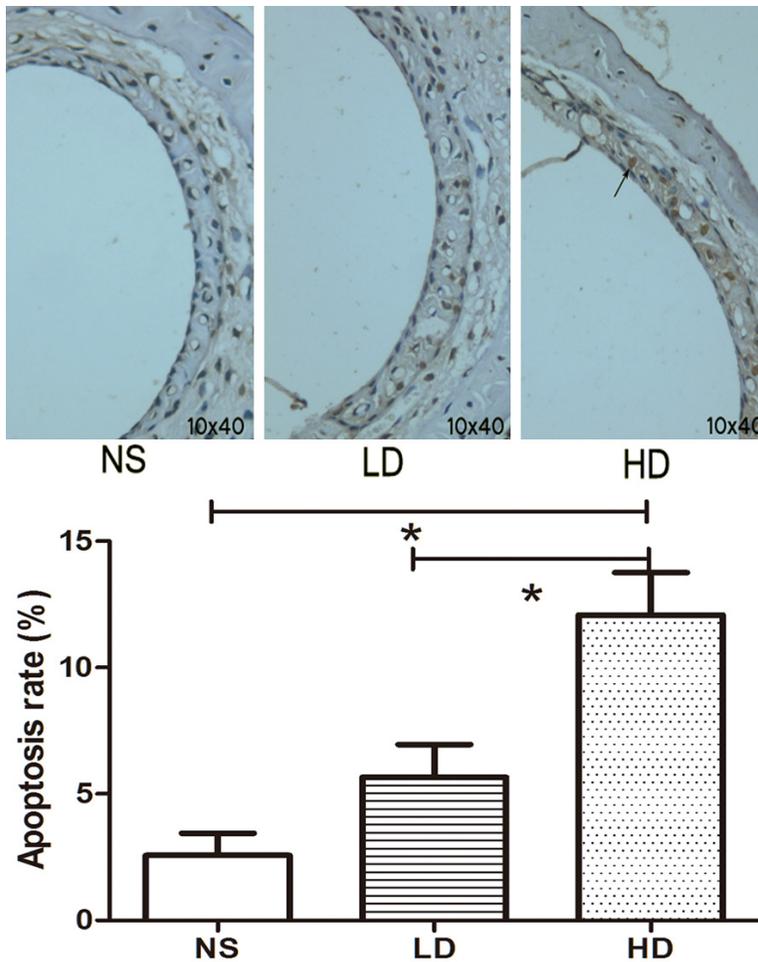


Figure 3. TUNEL staining (brown particles in the nucleus) showing the positive apoptotic cell located in the SV after CDDP application, especially in the HL group. The NS group exhibited no TUNEL-positive cells in the SV. Magnification: $\times 400$. NS: the group treated with normal saline; LD: the group treated with 3 mg/kg cisplatin; HD: the group treated with 6 mg/kg cisplatin. *, $p < 0.05$.

of SHC and the level of pSer36-P66Shc were measured with the corresponding antibodies and normalized to the level of β -actin that served as an internal reference. An equivalent of 20 μ g protein was separated by 10% SDS-PAGE (SDS-PAGE Kit) and then transferred to nitrocellulose membranes. SHC and pSer36-P66Shc levels were detected after probing the membranes with primary antibodies (SHC and β -actin 1:1000, pSer36-P66Shc 1:500), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5000) or goat anti-mouse (1:10000) immunoglobulin. Immunoreactivity was determined using the ECL Kit according to the manufacturer's instructions. The band density was

quantified using the ImageJ software (National Institutes of Health, USA).

Statistical analysis

All data are presented as the mean \pm standard deviation (Mean \pm SD). Statistical analyses were performed with the SPSS 16.0 software (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) was used for comparisons of the relative expression levels in different groups. The least significant difference (LSD) post hoc test was used to compare the differences between two groups. Differences with a P -value < 0.05 was considered to be statistically significant.

Results

Localization of p66Shc protein in the cochlea

Immunofluorescent labeling demonstrated that the p66-Shc was expressed only in the cytoplasm of the SV cells of the cochlea, and none in the organ of Corti (OC) cells or spiral ganglion (SG) cells of the cochlea (**Figure 1**).

CDDP-induced hearing loss

The auditory status was evaluated using ABR after the final administration of CDDP. As shown in **Table 1**, the ABR thresholds increased after CDDP-treatment. The significant increase occurred in the HD group ($P < 0.001$), as shown in **Table 1** and **Figure 2**.

CDDP-induced stria vascularis cell apoptosis

The positive apoptotic cells demonstrated by TUNEL staining were localized in the SV after CDDP application, especially in the HD group. The NS group did not exhibit any TUNEL-positive cells in the SV (**Figure 3**).

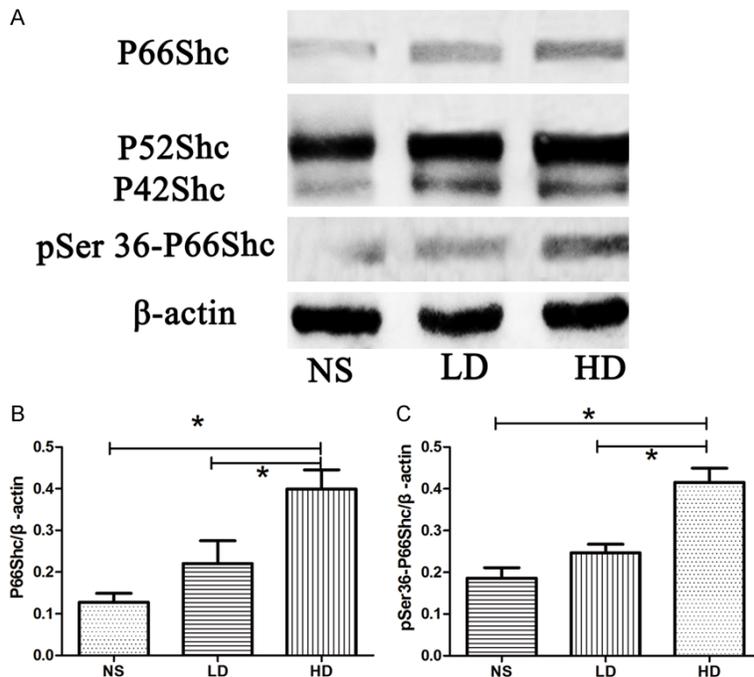


Figure 4. Western blot analysis: (A) Cisplatin induced an increase in the expression of p66Shc and pSer36-P66Shc. (B and C) Densitometry evaluations showed significant differences between the groups. NS: the group treated with normal saline; LD: the group treated with 3 mg/kg cisplatin; HD: the group treated with 6 mg/kg cisplatin. *, $p < 0.05$.

Increased protein levels of p66Shc and pSer36-P66Shc induced by CDDP

The result of Western blot assays showed that the protein level of p66Shc was significantly increased in the SV cells of the HD group than the other groups (HD group vs. NS group: $P < 0.001$, HD group vs. LD group: $P = 0.007$ compared to LD group) (Figure 4A and 4B). However, the relative changes in the total protein levels of p52Shc and p46Shc among the groups were not statistically significant ($P > 0.05$). The levels of Ser36-P-p66Shc were elevated in the SV cells of the HD group (HD group vs. NS group: $P < 0.001$, HD group vs. LD group: $P = 0.007$) (Figure 4A and 4C).

Discussion

Cisplatin-induced ototoxicity was first described by Rossof et al., in 1972. Since then, it has been broadly studied [14]. The ototoxicity is a bilateral, permanent, sensorineural type hearing loss that initially involves only the high frequencies and over a period also affects the low frequencies [15]. Similar to the results of the

previous studies in mice [16, 17], a significant increase in the ABR threshold was observed after CDDP treatment in the present study.

Cisplatin ototoxicity primarily occurs in the cochlea and is caused by apoptotic damage to the outer hair cells, spiral ganglion cells, and the peripheral cells of the SV [18]. The SV contains different epithelial and endothelial cells that maintain the ion composition of the endolymph, and thus, is known as the primary site of CDDP-induced ototoxicity [19]. In the present study, we demonstrated that induction of cell apoptosis in the SV after CDDP treatment by TUNEL assays was in agreement with previous findings in studies using systemic administration of CDDP [20, 21]. The SV, located on the lateral wall of the cochlea,

consists of three layers of cells: basal, middle, and marginal. These constitute a major component of the BLB party [22]. Furthermore, the SV is responsible for the generation and maintenance of the high positive EP and a high K^+ concentration in the endolymph of the cochlea, which is an energy source for hair cells to produce auditory current [23]. Any breakdown in the cellular integrity or increase in paracellular permeability (decoupling of tight junctions) between adjacent endothelial cells in the BLB rapidly induces the loss of EP with a consequent loss of hearing sensitivity [24]. Thus, the apoptosis of SV cells plays a major role in CDDP-induced ototoxicity.

p66Shc is the longest isoform of the ShcA family and varies from the other isoforms owing to the additional CH domain at the N-terminal, termed as the CH2 domain [25]. This CH2 domain contains a Ser36 residue, which is phosphorylated in response to oxidative stress and exerts a role in apoptosis [26]. Recently, p66Shc emerged as a key regulator of ROS production as well as a critical intracellular switch transmitting oxidative stress signals to DNA

damage in several cells [27-29]. The protein kinase C β (PKC β) can activate the p66Shc protein by phosphorylation of Ser36 residue, which results in a pS36-p66Shc product [30, 31]. Reportedly, pSer36-p66Shc is isomerized by the peptidyl-prolyl isomerase Pin1 and translocated to the mitochondrial intermembrane space [32]. Subsequently, the complex is recognized and dephosphorylated by the phosphatase PP2A [33]. In the intermembrane space of the mitochondria, p66Shc oxidizes cyt C, resulting in the generation of H₂O₂ via the transfer of electrons from the reduced cyt C to oxygen, thereby increasing the intracellular ROS level [34].

Furthermore, the current study showed that the p66Shc expressed in the cytoplasm of the cells in the SV of cochlea by immunofluorescent labeling. Moreover, the total protein levels of p66Shc and that of pSer36-p66Shc were significantly increased in the SV after HD CDDP-treatment. The increased levels of pSer36-p66Shc indicate a high oxidative stress in the tissue [35, 36]. Thus, the elevated levels of pSer36-p66Shc indicated an increased production of ROS in the SV of the CDDP-treated mice. This increased production of ROS is closely related to the CDDP-induced damage of the cochlea [37]. Therefore, our data indicated that cisplatin caused an increased expression of p66Shc and the activated form pSer36-p66Shc, which might be associated with cisplatin-induced ototoxicity via apoptosis of the SV cells.

In conclusion, we established a CDDP-induced hearing loss mouse model and demonstrated the occurrence of apoptosis in the SV cells of CDDP-treated mice, which might be associated with the increase and activation of p66Shc. Moreover, the SV was shown to be a primary site of the CDDP-induced ototoxicity. In addition, we infer that the p66Shc pathway is a main component of the mechanism underlying CDDP-induced ototoxicity.

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Disclosure of conflict of interest

None.

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