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Alpha-lipoic acid protects against cisplatin-induced ototoxicity via the regulation of MAPKs and proinflammatory cytokines

Jeongho Kim [a,b,1], Hyun-Ju Cho [a,1], Borum Sagong [a,b], Se-Jin Kim [c], Jae-Tae Lee [d], Hong-Seob So [c], In-Kyu Lee [e], Un-Kyung Kim [a,b,*], Kyu-Yup Lee [f,*], Yon-Sik Choo [a,b]

[a] Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea
[b] School of Life Sciences, KNU Creative BioResearch Group (BK21 plus project), Kyungpook National University, Daegu, Republic of Korea
[c] Vestibulocochlear Research Center, Center for Metabolic Function Regulation and Department of Microbiology, Wonkwang University School of Medicine, Iksan, Jeonbuk, Republic of Korea
[d] Department of Nuclear Medicine, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[e] Department of Internal Medicine, Research Institute of Aging and Metabolism, World Class University Program, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[f] Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Kyungpook National University, Daegu, Republic of Korea

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ABSTRACT

Cisplatin is an effective antineoplastic drug that is widely used to treat various cancers; however, it causes side effects such as ototoxicity via the induction of apoptosis of hair cells in the cochlea. Alpha-lipoic acid (ALA) has been reported to exert a protective effect against both antibiotic-induced and cisplatin-induced hearing loss. Therefore, this study was conducted to (1) elucidate the mechanism of the protective effects of ALA against cisplatin-induced ototoxicity using *in vitro* and *ex vivo* culture systems of HEI-OC1 auditory cells and rat cochlear explants and (2) to gain additional insight into the apoptotic mechanism of cisplatin-induced ototoxicity. ALA pretreatment significantly reduced apoptotic cell death of the inner and outer hair cells in cisplatin-treated organ of Corti explants and attenuated ototoxicity via marked inhibition of the increase in the expression of IL-1β and IL-6, the phosphorylation of ERK and p38, the degradation of IκBα, the increase in intracellular levels of ROS, and the activation of caspase-3 in cisplatin-treated HEI-OC1 cells. This study represents the first histological evaluation of the organ of Corti following treatment with ALA, and these results indicate that the protective effects of ALA against cisplatin-induced ototoxicity are mediated via the regulation of MAPKs and proinflammatory cytokines.

1. Introduction

Cisplatin (*cis*-diamminechloroplatinum II; CDDP) is a platinum-based compound that is widely used as a chemotherapeutic agent against a variety of human neoplasms [1]. However, the administration of high doses of cisplatin frequently causes significant side effects such as nephrotoxicity, neurotoxicity, and ototoxicity [2–5]. Indeed, 75–100% of patients who were administered cisplatin have been found to develop ototoxicity that was characterized by irreversible, progressive, bilateral, high-frequency, sensorineural hearing loss that is associated with tinnitus [6,7]. Several studies have revealed that the primary damage caused by cisplatin results from apoptotic cell death of the outer hair cells in the organ of Corti [8].

The cytotoxic mechanism of cisplatin has been shown to be associated with several factors. Specifically, the binding of cisplatin to guanine can result in apoptosis via the formation of inter- and intrastrand DNA crosslinks, aberrant genetic transcription, and cell cycle arrest [9]. Furthermore, mitochondrial dysfunction [10] and the increased generation of reactive oxygen species (ROS) [11] and reactive nitrogen species (RNS) [12] have been associated with cisplatin cytotoxicity. The other study demonstrated the role of cytokines in the pathogenesis of cisplatin cytotoxicity [13]. Cisplatin injection resulted the upregulation of cytokines and lead to severe defect in the kidney, and a treatment of salicylate reduces cisplatin nephrotoxicity accompanied with inhibition of TNF-α [14]. Interestingly, recent studies have reported that cisplatin-induced apoptotic cell death is caused by the expression and secretion of proinflammatory cytokines via the activation of ERK and NF-κB [15,16].

Alpha-lipoic acid (ALA) is an essential cofactor for mitochondrial respiratory enzymes that acts by scavenging free radicals,

* Corresponding authors. Address: Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea. Fax: +82 53 953 3066 (U.-K. Kim). Fax: +82 53 423 4524 (K.-Y. Lee).
E-mail addresses: kimuk@knu.ac.kr (U.-K. Kim), kylee@knu.ac.kr (K.-Y. Lee).
[1] These authors contributed equally to the work.

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chelating metal ions, and recycling antioxidants [17–20]. ALA has been introduced as a treatment for several diseases, including diabetes, polyneuropathy, cataracts, neurodegeneration, and nephropathies, based on its antioxidant activity [21,22]. Dose-dependent protection by ALA against cisplatin-induced ototoxicity was reported in an *in vivo* study [23]. Specifically, although auditory brainstem-evoked response (ABR) thresholds were elevated in rats treated with cisplatin alone, rats treated with cisplatin in combination with lipoic acid did not exhibit elevated ABR thresholds. Additionally, the protective effects of ALA occurred in a dose-dependent manner and were concurrent with changes in the concentrations of glutathione (GSH), lipid peroxidation, and antioxidant enzymatic activities in cisplatin-induced ototoxicity [23]. Although these reports demonstrated protective effects of ALA on cisplatin-induced hearing loss, cochlear hair cells were not histologically evaluated in this study, and little is known regarding the molecular mechanisms underlying the protective effects of ALA.

This study aimed at elucidates the mechanism of the protective effects of ALA in cisplatin-induced ototoxicity using both *in vitro* and *ex vivo* culture systems. We focused on the cytotoxic mechanism via the release of proinflammatory cytokines and the activation of MAPKs and NF-κB in cisplatin-induced cochlear injury and determined whether these apoptotic markers were altered following pretreatment with ALA in cisplatin-treated auditory cells.

2. Materials and methods

2.1. Culture of cochlear explants

Primary cochlear explants were prepared from postnatal day 3 (P3) Sprague Dawley rats. The dissected organ of Corti was incubated with high-glucose DMEM containing 10% FBS, at 37 °C and 5% CO_2 in a humidified incubator. After 16 h incubation, ALA treatment started 1 h before adding 20 μM cisplatin into culture media. The animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University and approved by the Committee on the Ethics of Animal Experiments of the Kyungpook National University (Permit number: 2013-34).

2.2. Histological evaluation

Cochlear explants were stained with Alexa Fluor® 555-conjugated phalloidin for 30 min. Then, specimens were mounted on glass slides using Fluoromount (Sigma, St. Louis, MO, USA) and examined by confocal microscopy (Zeiss LSM 700, Germany). The inner and outer hair cells were separately counted along the apical, middle, and basal turns in each cochlea for a quantitative assessment of hair cell loss. Only perfectly shaped hair cells in each region (with a length of 200 μm) were counted, and counting was repeated using five cochleae from each experimental group.

2.3. Immunohistochemical analysis and TUNEL (TdT-mediated dUTP nick end labeling) assay

The level of caspase-3 activation was evaluated using immunohistochemical analysis. Cisplatin-treated cochlear explants were immunostained with anti-active caspase-3 and Alexa Fluor® 488 Goat Anti-Rabbit IgG. Then, cochlear explants were stained with Alexa Fluor® 555-conjugated phalloidin and mounted on glass slides using Fluoromount. DNA fragmentation was also evaluated as a marker of apoptotic cell death using the TUNEL assay. ALA-treated cochlear explants were subjected to TUNEL assays according to the manufacturer's protocol (Promega, Madison, WI, USA). Cochlear explant specimens were visualized and the images were captured using a Zeiss DE/AX10 Imager A1 fluorescence microscope system (Carl Zeiss, Oberkochen, Germany).

2.4. HEI-OC1 cell culture

The establishment and characterization of the HEI-OC1 auditory cell line have been described by Kalinec et al. [24]. HEI-OC1 cells were maintained in high-glucose DMEM containing 10% FBS at 33 °C and 10% CO_2 in a humidified incubator.

2.5. MTT assay

HEI-OC1 cells (0.3×10^5 cells per well of a 24-well plate) were plated and incubated for 16 h. Cells were treated with varying doses of cisplatin for 30 h with or without pretreatment with 1 mM ALA for 1 h. Cell viability was evaluated using MTT assay as described previously [15].

2.6. Determination of ROS levels

The effects of cisplatin and ALA on the intracellular ROS levels in HEI-OC1 cells were determined using DCFH-DA assays (DCFH-DA; Invitrogen-Molecular Probes, Eugene, OR, USA). Briefly, HEI-OC1 cells were cultured for 16 h and were then treated with 20 μM cisplatin for 24 h with or without pretreatment with 1 mM ALA. Subsequently, the cells were harvested using trypsin–EDTA into 1.5 mL tubes and were incubated with 10 μM DCFH-DA/PBS for 30 min. Flow cytometric analyses (10,000 events per sample) of DCFH-DA-incubated cells were then performed using a BD FACS Aria III flow cytometer (BD Biosciences, San Diego, CA, USA) using excitation and emission wavelengths at 485 and 538 nm, respectively, and were then evaluated using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.7. Western blot analysis

HEI-OC1 cells were harvested and resuspended in RIPA buffer (Elpis Biotech, Daejeon, Korea) containing the Protease Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA, USA). The cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting, as previously described [15].

2.8. RNA isolation and reverse-transcription PCR

Total RNA was extracted from HEI-OC1 cells using an RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Single-stranded cDNA was then synthesized from the total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Direct PCR using PrimeSTAR® Taq polymerase (Takara Shuzo, Kyoto, Japan) was conducted according to the manufacturer's protocols, and 5 μL of each PCR product was separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The primer sequences used for PCR amplification were as follows: IL-1β F, AAG GAG ACC AAG CAA CGA C; IL-1β R, GAG ATT GAG CTG TCT GCT CA; IL-6 F, TTG CCT TCT TGG GAC TGA TGC; and IL-6 R, TTG GAA ATT GGG GTA GGA AGG A.

2.9. Reagents

Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bro-

mide (MTT; Sigma, St. Louis, MO, USA), ALA (Thioctacid inj®, Bukwang Pharmaceutical Co., Ltd., Seoul, Korea), and cisplatin (Cispuran®, Dong-A pharmaceutical Co., Ltd., Seoul, Korea) were used in this study. For Western blot and immunohistochemical analysis, rabbit anti-IκBα, rabbit anti-active caspase-3, rabbit anti-p38, rabbit anti-ERK, rabbit anti-phospho-p38, rabbit anti-phospho-ERK, and goat anti-rabbit antibodies (Cell Signaling Technology, Beverly, MA, USA) as well as Alexa Fluor® 488 goat anti-rabbit IgG (H + L) and Alexa Fluor® 555-conjugated phalloidin (Invitrogen-Molecular Probes, Eugene, OR, USA) were used.

2.10. Statistical analysis

Statistical analysis was performed using independent *t*-test and one-way ANOVA, and each experiment was performed independently and repeated at least three times. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Alpha-lipoic acid protects cochlear explants from cisplatin-induced apoptosis

To investigate the protective effects of ALA against cisplatin in cochlear tissues, primary cultures of cochlear explants were exposed to cisplatin, and the hair cells at the apical turn, middle turn, and basal turn were subsequently counted. The cochleae that were treated with 20 μM cisplatin exhibited disarrayed hair cells and significantly disrupted stereocilia bundles (Fig. 1A). The stereocilia of the OHCs exhibited more severe defects than those of the IHCs, and the OHCs exhibited regional variation in their susceptibility to cisplatin, with gradual severity of hair cell loss from the apical to the basal turn. However, the group cotreated with 1 mM ALA exhibited an orderly arrangement of hair cells consisting of three rows of OHCs and one row of IHCs, indicating a V-shaped OHC stereocilia arrangement that was similar to the control and ALA alone group (Fig. 1A). The numbers of intact hair cells on the apical turn, middle turn, and basal turn are shown in Fig. 1B. The group cotreated with 1 mM ALA exhibited significantly less hair cell loss in comparison to the group treated with cisplatin alone.

Apoptosis of the damaged hair cells of cisplatin-treated organ of Corti explants was evaluated both by immunohistochemistry using an anti-active caspase-3 antibody and by TUNEL assays, which labeled the cleaved caspase-3 and DNA fragments in the apoptotic cells, respectively. Although the activation of caspase-3 was markedly increased in the cisplatin-treated (20 μM) group, pretreatment with ALA (1 mM) resulted in decreased activation of caspase-3 (Fig. 2A). Although the groups treated with only ALA (1 mM) also exhibited a tendency for increased caspase-3 activation in comparison to the control group, the hair cell morphology

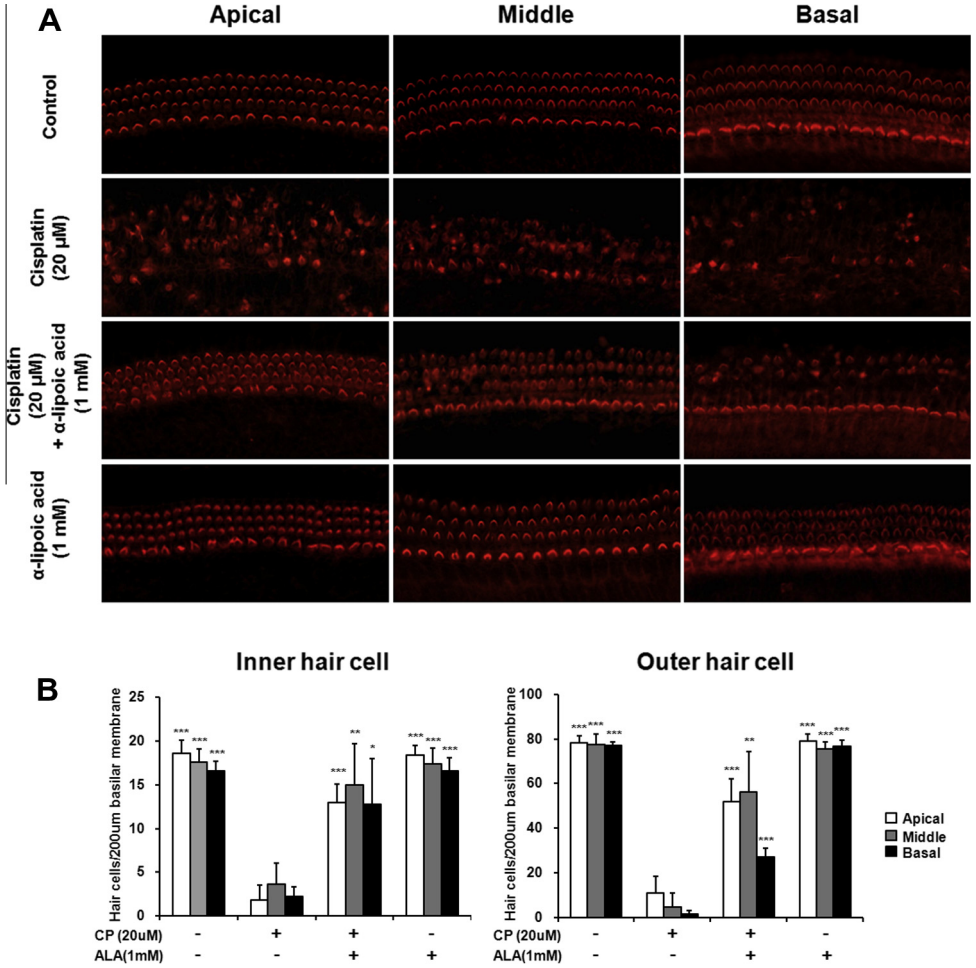

Fig. 1. Effect of ALA on cisplatin-induced ototoxicity in organ of Corti explants. Organ of Corti explants were treated with culture media, 20 μM cisplatin, 20 μM cisplatin with 1 mM ALA pretreatment, or 1 mM ALA alone for 30 h. (A) The explants were stained with Alexa Fluor® 555-conjugated phalloidin and were then observed using confocal microscopy. (B) The average number of hair cells/200 μm basilar membrane along the apical, middle, and basal turns of the cochleae are shown. The results are representative of five independent experiments. The data represent the means ± S.D. from five independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with cisplatin alone as determined using an independent *t*-test. ALA, alpha-lipoic acid; CP, cisplatin.

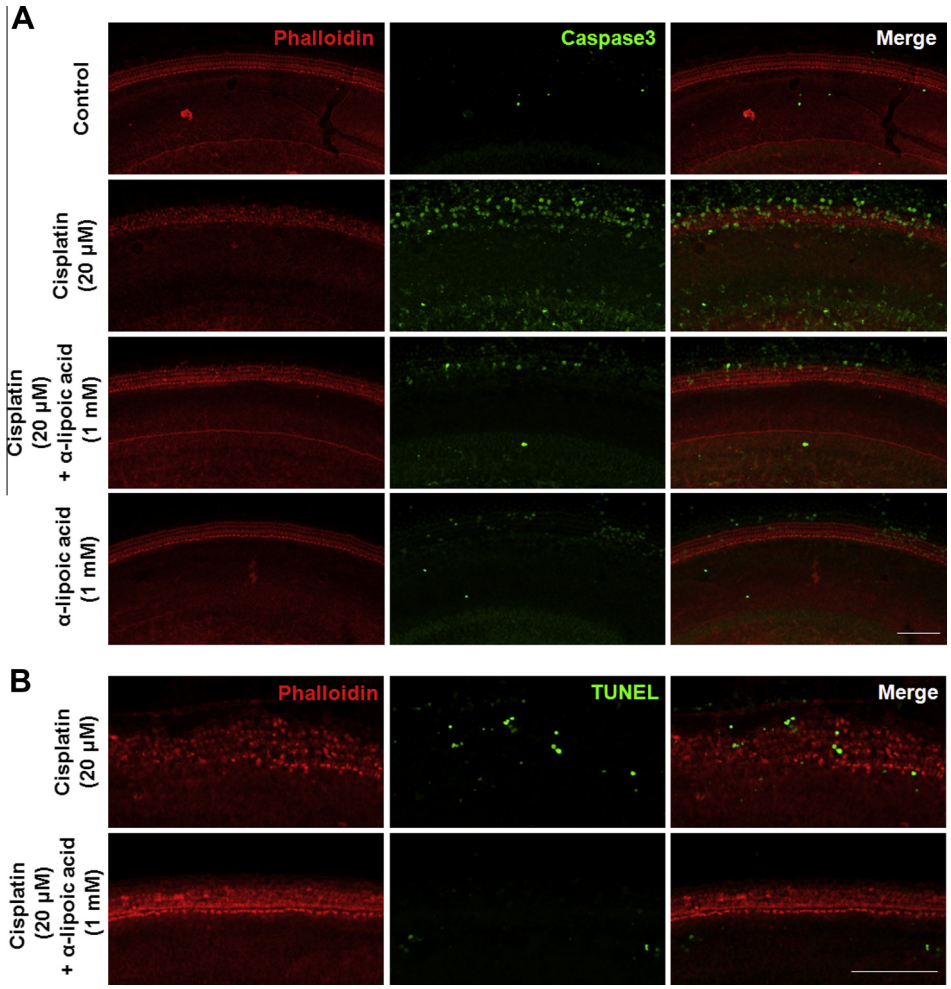

Fig. 2. Effect of ALA on cisplatin-induced apoptotic cell death in organ of Corti explants. Organ of Corti explants were treated with culture media, 20 μM cisplatin, 20 μM cisplatin with 1 mM ALA pretreatment, or 1 mM ALA alone for 30 h. Apoptotic cells in the organ of Corti were evaluated using active caspase-3 (A) and TUNEL assays (B). The middle turns of the cochlea were stained with Alexa Fluor 555-conjugated phalloidin (red). Active caspase-3 and TUNEL signal were labeled with green. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was not altered. TUNEL assays also revealed a protective effect of ALA against cisplatin-induced apoptosis in cochlear explants (Fig. 2B).

3.2. Alpha-lipoic acid attenuates cisplatin-induced apoptosis in HEI-OC1 cells and prevents the activation of IκBα

To assess the protective effects of ALA on cisplatin cytotoxicity, cell viability was assessed using MTT assays in HEI-OC1 cells that were treated with varying concentrations of cisplatin and ALA. Cisplatin treatment significantly decreased cell viability in a dose-dependent manner. Cells treated with 20 μM cisplatin exhibited 39.45% cell viability; therefore, this cisplatin concentration was selected to investigate the effects of cisplatin with ALA pretreatment (Fig. 3A). A 1 h treatment using varying concentrations of ALA prior to 20 μM cisplatin treatment significantly elevated the cell viabilities in comparison to treatment with cisplatin alone (20 μM). The protective effect of ALA against cisplatin cytotoxicity increased in a dose-dependent manner, with the maximum protective effect observed in response to a 2 mM ALA pretreatment in HEI-OC1 cells (Fig. 3B).

At the western blot assay from HEI-OC1 cells treated with 20 μM cisplatin for 24 h, the activation of caspase-3 was observed, but the cells pretreated with ALA before cisplatin treatment did not exhibit any caspase-3 activation (Fig. 3C). The total expression level of IκBα, which is the inhibitory binding protein of NF-κB, decreased in a time-dependent manner in HEI-OC1 cells treated with 20 μM cisplatin while the expression levels of IκBα remained unaltered in HEI-OC1 cells pretreated with 1 mM ALA (Fig. 3C). These results indicated that cisplatin decreased the total levels of IκBα (*i.e.*, increased the levels of phosphorylated IκBα), which resulted in apoptosis of HEI-OC1 cells, whereas pretreatment with 1 mM ALA prevented the phosphorylation of IκBα and the activation of caspase-3, thereby preventing cisplatin-induced apoptosis of HEI-OC1 cells.

3.3. Alpha-lipoic acid attenuates cisplatin-induced ROS generation in HEI-OC1 cells

We measured the intracellular levels of ROS generated by HEI-OC1 cells in response to cisplatin and ALA using the fluorescent probe DCFH-DA. As shown in Fig. 4A, HEI-OC1 cells treated with 20 μM cisplatin exhibited markedly increased fluorescence intensity in comparison to the control group. Pretreatment with 1 mM ALA for 1 h markedly reduced the cisplatin-induced generation of ROS in HEI-OC1 cells (Fig. 4A). The mean probe intensity in comparison to that of the control group is shown in Fig. 4B. Treatment with 20 μM cisplatin increased the fluorescence intensity by 54%,

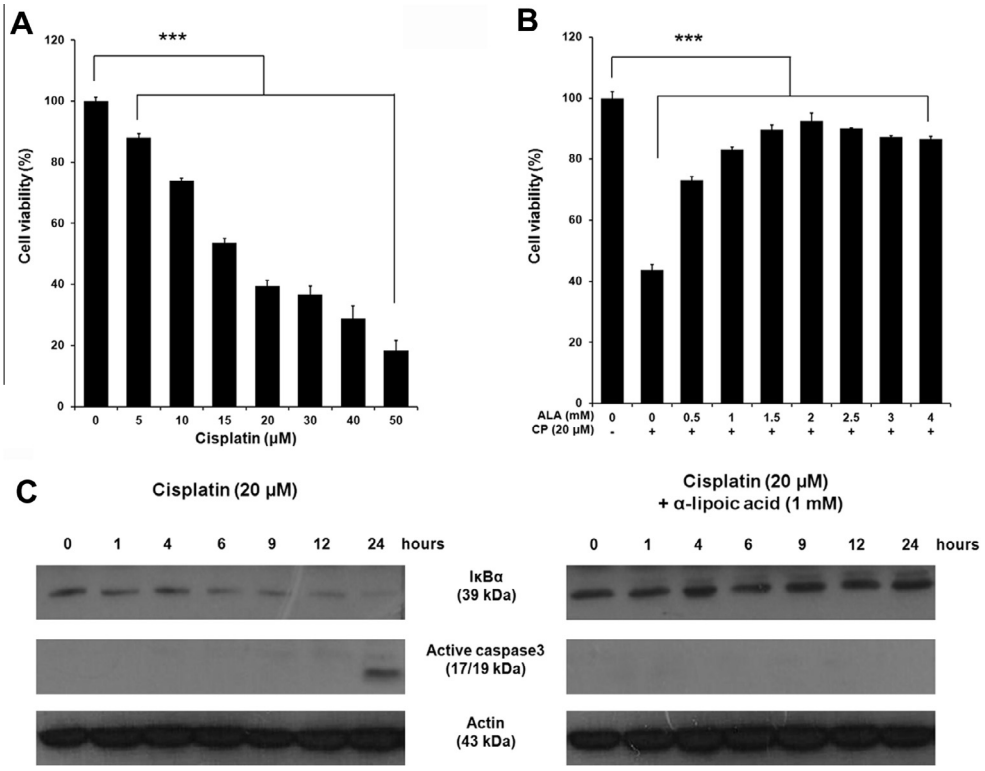

Fig. 3. Effects of ALA on the viability, IκBα level, and activation of caspase-3 in HEI-OC1 cells after treatment with cisplatin. Cell viability was measured by MTT assay. (A) HEI-OC1 cells were treated with various concentrations of cisplatin for 30 h. (B) HEI-OC1 cells were pre-treated with varying doses of ALA followed by 20 μM of cisplatin for 30 h. The data represent the means ± S.D. of three independent experiments performed in duplicate. ***$P < 0.001$ (determined using an independent *t*-test). (C) HEI-OC1 cells were treated with 20 μM cisplatin for various lengths of time with or without 1 mM ALA pretreatment for 1 h. Intracellular proteins were analyzed for IκBα, active caspase-3, and actin using Western blot analysis. ALA, alpha-lipoic acid; CP, cisplatin.

whereas pretreatment with 1 mM ALA did not alter the fluorescence, and treatment with 1 mM ALA alone resulted in a 14% decrease in the fluorescence intensity.

3.4. Alpha-lipoic acid inhibits the increase in proinflammatory cytokines and the activation of MAPKs induced by cisplatin treatment in HEI-OC1 cells

RT-PCR analysis indicated increased IL-6 and IL-1β mRNA levels 24 h after cisplatin treatment. However, pretreatment with ALA for 1 h resulted in a significant decrease in the mRNA expression of IL-6 (22%) and IL-1β (38%) in comparison to treatment with cisplatin alone (Fig. 4C). Subsequently, we examined the effects of ALA on ERK and p38 activation in cisplatin-treated HEI-OC1 cells using Western blot analysis (Fig. 4D). Exposure of cells to 20 μM cisplatin induced the phosphorylation of ERK (129%) and p38 (318%) at 24 h in comparison to the control group. Pretreatment of cells with ALA for 1 h prior to cisplatin treatment induced a marked reduction in the phosphorylation of ERK and p38 of approximately 54% and 45%, respectively, in comparison to the cisplatin-treated group.

4. Discussion

Many therapeutic medicines such as ALA [19–22,25,26], D-methionine [27,28], NAC [29,30], and sodium thiosulfate [28,31] have been introduced to prevent cisplatin-induced cytotoxicity. Among these, ALA is well known due to its protective effects in auditory organs; however, the histology of ALA-treated organ of Corti and hair cells has not yet been investigated [23,32–34]. In the present study, we identified the protective effects of ALA against cisplatin induced ototoxicity in organ of Corti explants and observed the morphology of hair cells. Disarrayed hair cells with significantly disrupted stereocilia bundles from both inner and outer hair cells and typical base-to-apex progressive defects of OHCs were observed in 20 μM cisplatin-treated cochlear explants. Although pretreatment with 1 mM ALA for 1 h prior to 20 μM cisplatin treatment prevented the loss of most IHCs and some OHCs, OHC defects remained at the basal turn. This incomplete protection is thought to be caused by differences in the severity of hair cell defects caused by cisplatin treatment rather than from differences in the sensitivity to ALA based on the region of the organ of Corti. These results are consistent with those of a study conducted by Rybak et al., who found similar ABR thresholds at all frequencies in response to varying doses of ALA and cisplatin in rats [23].

Pretreatment with 2 mM ALA prevented most inner and outer hair cell death but resulted in abnormal shaped stereocilia rather than the normal V-shaped stereocilia (data not shown), which may be related to the cytotoxicity of ALA in several cancer cells [25,35,36]. However, additional studies are required to clearly define the stereocilia abnormality observed in cochlear explants in response to treatment with 2 mM ALA. Lipoic acid has been reported to be toxic to SW620 HFST cells, with LC_{50} values of approximately 3 mM [37]. Therefore, clinical trials should be performed to evaluate the safety of ALA at high doses for clinical treatment in humans.

Several signaling pathways are altered in cisplatin-induced apoptosis, including the perturbation of the redox status, increased lipid peroxidation, the formation of DNA adducts, and an increased production of proinflammatory cytokines. We focused on proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, which were increased through the activation of MAPKs and NF-κB in cisplatin-treated HEI-OC1 cells. Previous studies have demonstrated

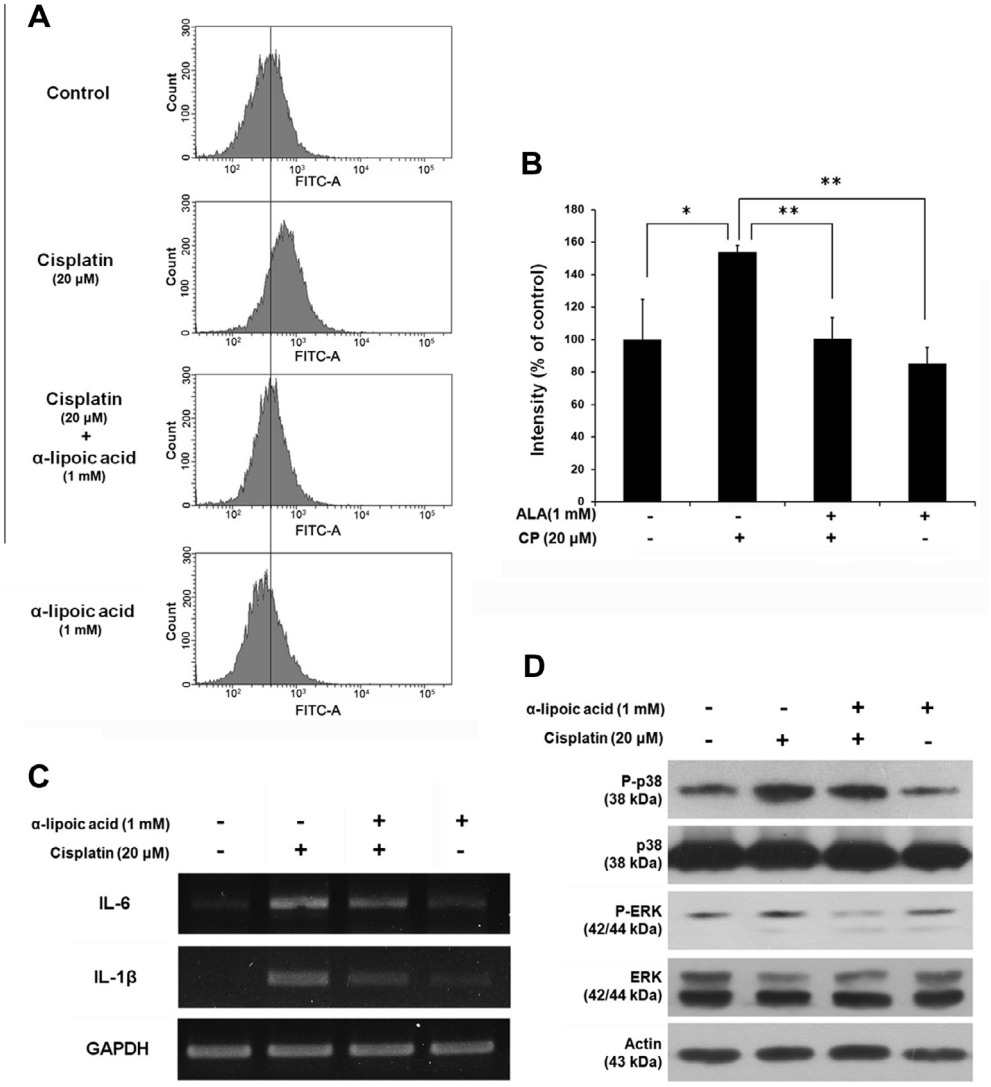

Fig. 4. Effects of ALA on intracellular ROS, proinflammatory cytokines and activation of MAPKs in cisplatin-treated HEI-OC1 cells. HEI-OC1 cells were treated with media, 20 μM cisplatin, 20 μM cisplatin with 1 mM ALA pretreatment, or 1 mM ALA alone for 24 h. (A) The levels of intracellular ROS were measured by flow cytometry using a peroxide-sensitive fluorescent probe, DCFH-DA. (B) The fluorescence levels of each group were compared and are represented by a bar graph. The data represent the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with cisplatin alone as determined by an independent *t*-test. (C) The mRNA expression levels of proinflammatory cytokines were analyzed. IL-1β, IL-6, and GAPDH cDNAs were amplified by RT-PCR using specific primer sets. (D) The intracellular proteins were analyzed for p38, ERK, P-p38K, P-ERK, and actin using Western blot analysis. ALA, alpha-lipoic acid; CP, cisplatin.

that neutralization of proinflammatory cytokines and pharmacological inhibition of MAPKs can significantly decrease the cytotoxicity of cisplatin-treated HEI-OC1 cells [15]. Protective effects of ALA resulting from significant decreases in the levels of proinflammatory cytokines that were markedly increased by LPS administration in rats have also been reported [26]. Based on these preliminary findings, we investigated whether ALA could decrease the expression levels of proinflammatory cytokines that are induced by cisplatin (thereby resulting in anti-ototoxicity in cisplatin-treated auditory cells) by determining the levels of these cisplatin-induced biomarkers of ototoxicity after pretreatment with ALA. Interestingly, our results indicated that ALA attenuated ototoxicity in cisplatin-treated HEI-OC1 cells by inhibiting increases in IL-1β and IL-6 expression, inhibiting the phosphorylation of ERK and p38, preventing the degradation of IκBα, and preventing increased levels of intracellular ROS and the activation of caspase-3.

This study represents the first histological evaluation of the organ of Corti following ALA treatment. These results demonstrated that the protective mechanism of ALA in cisplatin-induced ototoxicity is mediated via the regulation of proinflammatory cytokines and the activation of MAPKs.

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