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Anti-apoptotic effect of phloretin on cisplatin-induced apoptosis in HEI-OC1 auditory cells

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Abstract:
Cisplatin is a highly effective chemotherapeutic agent, but it has significant ototoxic side effects. Apoptosis is an important mechanism of cochlear hair cell loss following exposure to cisplatin. The present study examined the effects of phloretin, a natural polyphenolic compound found in apples and pears, on cisplatin-induced apoptosis. We found that phloretin induced the expression of heme oxygenase-1 (HO-1) protein in a concentration- and time-dependent manner. Phloretin induced nuclear factor-E2-related factor 2 (Nrf2) nuclear translocation, and dominant-negative Nrf2 attenuated phloretin-induced expression of HO-1. Phloretin activated the JNK, ERK and p38 mitogen-activated protein kinase (MAPK) pathways, and the JNK pathway played an important role in phloretin-induced HO-1 expression. Phloretin protected the cells against cisplatin-induced apoptosis. The protective effect of phloretin was abrogated by zinc protoporphyrin IX (ZnPP IX), a HO inhibitor. Furthermore, phloretin pretreatment inhibited mitochondrial dysfunction and the activation of caspases. These results demonstrate that the expression of HO-1 induced by phloretin is mediated by both the JNK pathway and Nrf2; the expression inhibits cisplatin-induced apoptosis in HEI-OC1 cells.

Key words:
phloretin, cisplatin, heme oxygenase-1, JNK, caspase

Abbreviations: DMEM – Dulbecco's modified Eagle's medium, FBS – fetal bovine serum, HEI-OC1 – House Ear Institute-Organ of Corti 1, HO-1 – heme oxygenase-1, MAPKs – mitogen-activated protein kinases Nrf2 – nuclear factor-E2-related factor 2, PKC – protein kinase C, ZnPP – zinc protoporphyrin

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II, CDDP) is a highly effective chemotherapeutic agent used to treat several types of solid tumors [15]. However, reversible and irreversible side effects, including ototoxicity and nephrotoxicity, can limit its utility and therapeutic profile [3, 20]. Therefore, many researchers have tried to ameliorate the ototoxic side effect of cisplatin. The loss of hearing appears to result from the destruction of outer hair cells in the organ of Corti as a result of apoptosis [12]. Several studies have indicated that antioxidants are effective in the prevention of drug-induced hearing loss [4, 33]. Indeed, antioxidants have shown efficacy in the attenuation of noise-induced hearing loss, protecting auditory outer hair cells and electrophysiological responsiveness [33].

Heme oxygenase (HO)-1 is a critical factor in response to oxidative injury, a major result of which is the degradation of heme to biliverdin, iron, and car-

bon monoxide [24, 36]. Previous studies have demonstrated that HO-1 works as a part of the cytoprotective mechanism, which includes antioxidant [10], anti-inflammatory [17, 23], antiproliferative [11], and antiapoptotic properties [8]. Thus, considering the cytoprotective role of HO-1, the induction of HO-1 expression by pharmacological modulation may represent a novel target for therapeutic treatments of various diseases.

Phloretin, a natural polyphenolic compound found in apples and pears, has been shown to exert antitumor activity through inhibition of protein kinase C (PKC) activity and induction of apoptosis [25]. Phloretin has been reported to be a hepatoprotective agent, with studies showing that it prevents tacrine-induced cytotoxicity in human liver cancer cells [2]. Phloretin has potent antioxidant activity and antiproliferative effects on cancer cells [13, 31]. However, little is known about the protective mechanism by which phloretin rescues cells from oxidative stresses. Recently, attention has been focused on the effect of HO-1 induced by phytochemicals investigating the role of HO-1 against oxidative cell damage [30]. In this study, our aim was to elucidate the molecular mechanism of phloretin protection against cisplatin-induced apoptosis in HEI-OC1 cells, specifically focusing on the upregulation of HO-1.

Materials and Methods

Materials

Phloretin and cisplatin were from Sigma-Aldrich (St. Louis, MO, USA). Zinc protoporphyrin IX (ZnPP IX), an inhibitor of heme oxygenase activity, was purchased from Porphyrin Products (Logan, UT, USA). PD098059, SB203580, SP600125, and Anti-HO-1 antibody were purchased from Calbiochem (San Diego, CA, USA). Antibodies against Bcl-2, Bax, and Nrf2 were purchased from Santa Cruz Biotechonology (Santa Cruz, CA, USA). Anti- phospho-JNK, -ERK, and -p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Unless indicated otherwise, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The establishment and characterization of the conditionally immortalized House Ear Institute-Organ of Corti 1 (HEI-OC1) cells were described by Kalinec et al. [22]. HEI-OC1 cells have recently been established from long-term cultures of Immorto-mouse cochlea and characterized. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, NY, USA) at 33°C in a humidified incubator with 5% CO_2.

Cell viability

Cells were subcultured in 96-well plates at a density of 5×10^4 cells/well. Cells were treated with cisplatin in the presence or absence of phloretin or ZnPP. The MTS assay was performed with the CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega Corp., WI, USA), according to the manufacturer's instructions. The absorbance was read at 490 nm on an ELISA reader, and the percentage of cell survival was determined.

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear fractions were prepared as previously described [32]. Briefly, cells were washed three times with cold PBS and centrifuged at 130 × g for 10 min. The pellet was carefully resuspended in 3 pellet volumes of cold buffer containing 20 mM HEPES, pH 7.0, 0.15 mM EGTA, 10 mM KCl, 1% Nonidet-40, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4. The homogenate was then centrifuged at 500 × g for 20 min, and the nuclear pellet was washed in 5 pellet volumes of cold PBS. After centrifugation at 500 × g for 20 min, nuclei were resuspended in 2 pellet volumes of cold hypertonic buffer containing 10 mM HEPES, pH 8.0, 25% glycerol, 0.4 M NaCl, 0.1 mM EDTA, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4 and incubated for 30 min at 4°C on a rotating wheel. Nuclear debris was removed by centrifugation at 900 × g for 20 min at 4°C. The supernatant was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blot analysis with anti-Nrf2 and anti-Lamin B antibodies.

Transient transfection

A day before transfection, cells were subcultured at a density of 1×10^6 cells in a 60 mm dish to maintain approximately 70–80% confluency. The cells were transiently transfected using lipofectamine with a plasmid containing dominant-negative (DN) JNK, according to the manufacturer's instructions (GIBCO-BRL, NY, USA). After overnight transfection, cells were treated 10 μM phloretin for 12 h and proteins were analyzed by Western blot analysis.

TUNEL assay

Cells were grown on glass cover slips for 24 h. Cells were treated with cisplatin for 36 h and then subjected to a TUNEL assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 solution in PBS for 5 min, and incubated with fluorescein-12-dUTP and Terminal Doxynucleotidyl Transferase in the dark for 60 min. Cells were analyzed under a fluorescence microscope.

Western blot analysis

Western blot analysis was performed as follows. Briefly, cells were harvested, washed twice with ice-cold PBS, and resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin). Protein concentration was determined with the Lowry protein assay kit (BIO-RAD Laboratories, Hercules, CA, USA). Samples were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membranes were incubated for 1 h in the presence of primary antibodies and then incubated for 1 h with secondary antibodies. The protein bands were visualized using chemiluminescent reagents according to the manufacturer's instructions (Supersignal Substrate; Pierce, USA). All densitometric values obtained for the protein were normalized to values for β-actin obtained on the same blot. The protein level in treated cells was expressed in densitometric absorbance units, normalized to control untreated samples, and expressed as fold induction compared to controls.

Caspase activity assay

Apoptosis was assessed by analysis of activation of caspase-8, -9, and -3 using Caspase-8, -9, and -3 assay kits (R&D System, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analysis

Differences in the data among the groups were analyzed by one-way ANOVA, and all values were expressed as the mean ± SD. The differences between groups were considered to be significant at $p < 0.05$.

Results

Phloretin induces HO-1 expression in HEI-OC1 cells

HEI-OC1 cells were treated with various concentrations of phloretin (1–20 μM) for 18 h. As shown in Figure 1A, phloretin induced the expression of HO-1 in a dose-dependent manner. The maximal induction of HO-1 protein was achieved at 10 μM phloretin (Fig. 1C). The induction of HO-1 was also time dependent (Fig. 1B). Expression of HO-1 was evident as early as 6 h and reached a maximum 24 h after treatment with 10 μM phloretin (Fig. 1D).

Phloretin increases Nrf2 nuclear translocation

The transcription factor Nrf2 plays an essential role in expression of antioxidant enzymes and in the activation of other stress-inducible genes in response to oxidative stress [21]. Therefore, we investigated whether treatment with phloretin induces the translocation of Nrf2 to the nuclei of HEI-OC1 cells. The cells were treated with 10 μM phloretin for 0.5, 1, 2, and 4 h. At each time point, the level of Nrf2 protein was determined by Western blotting. Phloretin induced the accumulation of Nrf2 in the nuclei (Fig. 2A–C). When the cells were transfected with a dominant-negative mutant plasmid of Nrf2 (Nrf2 DN), the induction of HO-1 protein by phloretin was mostly suppressed (Fig. 2D and E).

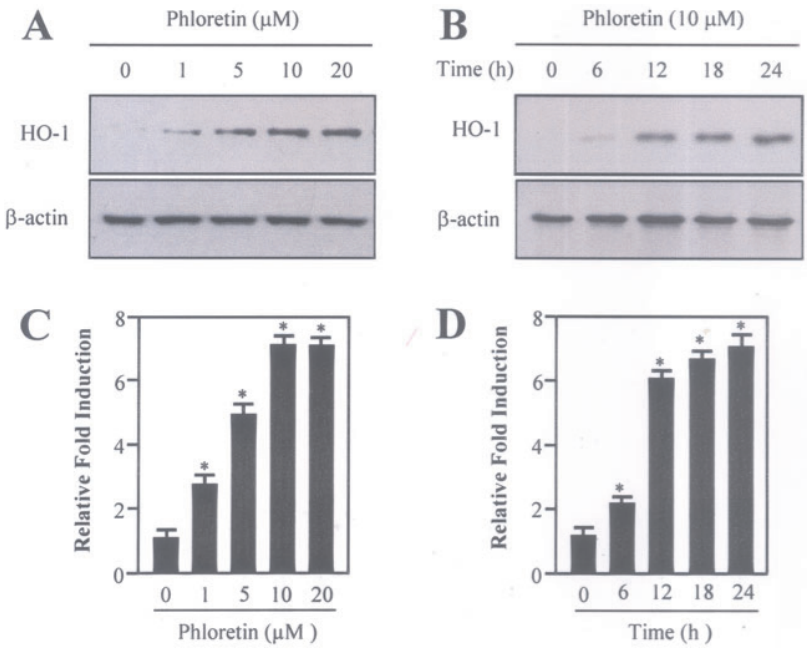

Fig. 1. Induction of HO-1 expression by phloretin in HEI-OC1 cells. (**A** and **C**) HO-1 expression was measured in HEI-OC1 cells 24 h after treatment with various concentrations of phloretin. (**B**) and **D**) Cells were treated with 10 μM phloretin, and HO-1 expression was measured at time points indicated in the figure. Total cellular proteins were isolated from cells treated with phloretin and Western blot analysis was performed using specific antibodies for HO-1 and β-actin. (**C** and **D**) Relative fold induction of HO-1 protein levels was quantified as described under "Materials and Methods". Data shown are the results of 1 of 3 independent experiments

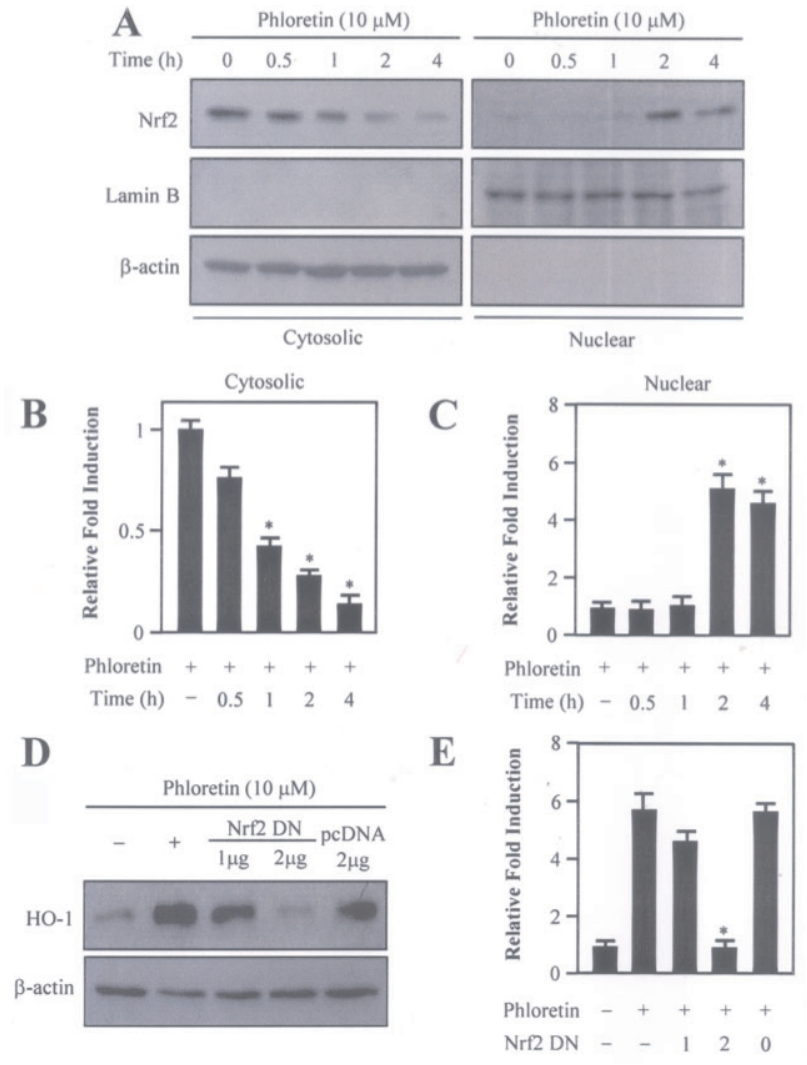

Fig. 2. Involvement of Nrf2 is essential in the process of HO-1 expression induced by phloretin. (**A**-**C**) HEI-OC1 cells were treated with 10 μM phloretin for various times, as indicated in the figure. Nrf2 protein in the cytosol and the nuclei were detected by Western blotting. (**D** and **E**) HEI-OC1 cells were transfected with empty vector (pcDNA3) and expression vector for the dominant-negative of Nrf2 (Nrf2 DN). After 16 h of transfection, cells were kept in low serum medium and were stimulated with 10 μM phloretin for 24 h. Cellular proteins were isolated, and Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Relative fold induction of Nrf2 and HO-1 protein levels was quantified as described under "Materials and Methods". Data show the results of 1 of 3 independent experiments. * $p < 0.05$ *vs.* phloretin-treated cells

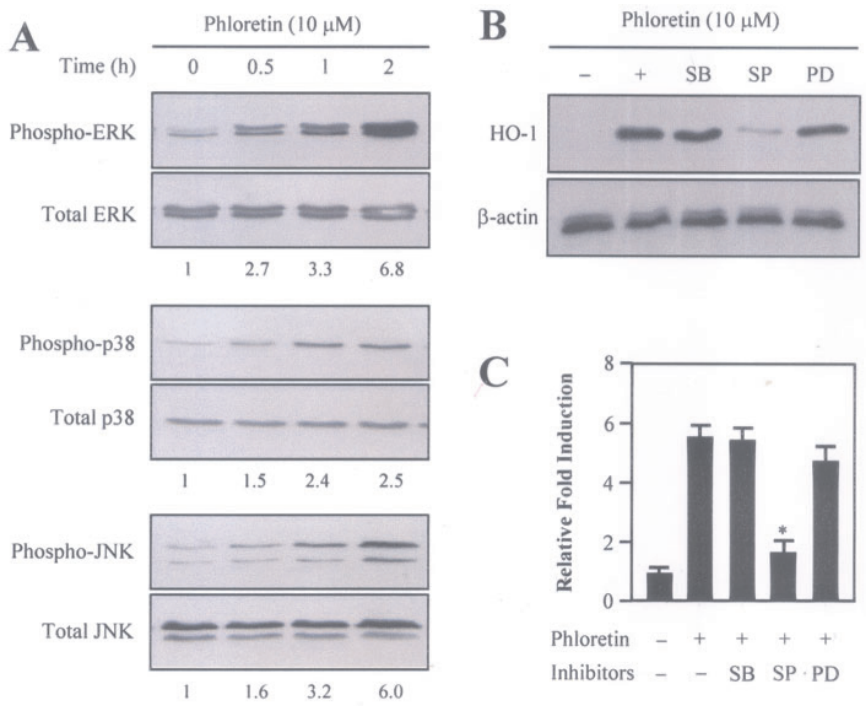

Fig. 3. Effect of phloretin on phosphorylation of MAPKs in HEI-OC1 cells. (**A**) The cells incubated with 10 μM phloretin for the indicated times were subjected to Western blot analysis using anti-phospho JNK, ERK, or p38 antibodies. As controls, the same cell lysates were subjected to Western blot analysis using total JNK, ERK, or p38 antibodies. (**B** and **C**) Cells were pretreated with or without SB203580 (SB), SP600125 (SP), or PD98059 (PD) and then incubated in the absence or presence of 10 μM phloretin for 24 h. Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Relative fold induction of MAPKs activation and HO-1 protein levels was quantified as described under "Materials and Methods". The data shown are from 1 of 3 independent experiments. * $p < 0.05$ *vs.* phloretin-treated cells

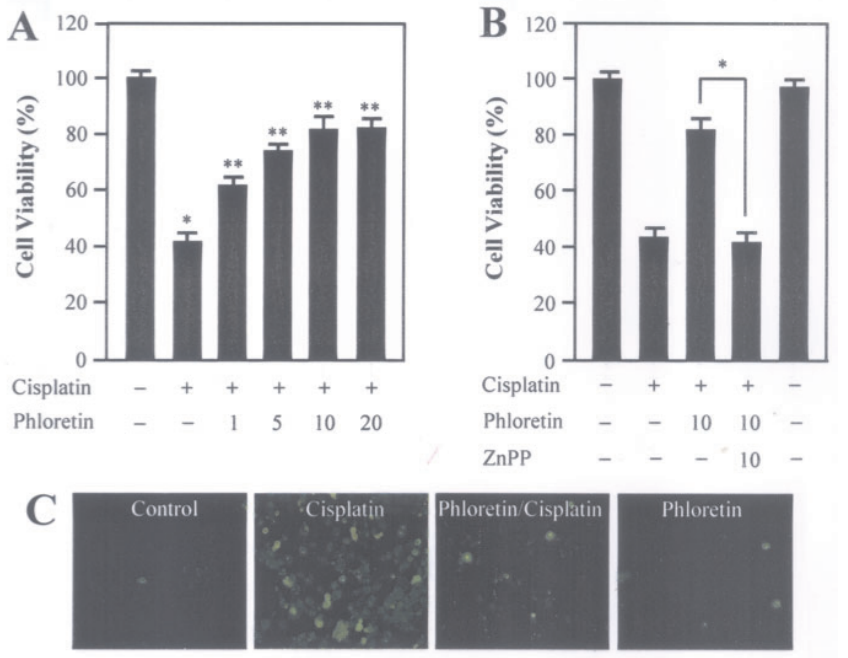

Fig. 4. The protective effect of phloretin on cisplatin-induced apoptosis in HEI-OC1 cells. (**A**) Cells were pretreated with the indicated doses of phloretin for 12 h and then incubated with 20 μM cisplatin for 36 h. (**B**) Cells were pretreated with 10 μM phloretin for 12 h in the absence or presence of 10 μM ZnPP and were then incubated with 20 μM cisplatin for 36 h. Cell viability was measured using the MTS assay. Data represent the means ± SD of three independent experiments. * $p < 0.05$ *vs.* control; ** $p < 0.05$ *vs.* cisplatin-treated cells. (**C**) HEI-OC1 cells were pretreated with 10 μM phloretin for 12 h, incubated with 20 μM cisplatin for 36 h and then subjected to the TUNEL assay

Phloretin-induced expression of HO-1 is mediated by the JNK pathway

Several studies on the regulation of HO-1 expression have focused on the roles of the MAPK pathways in various cell culture systems [14, 18, 26]. Therefore, we tested whether phloretin-induced HO-1 expression occurs through the action of the MAPK pathway in HEI-OC1 cells. The results showed that the phosphorylation of JNK, ERK, and p38 were all increased by phloretin in HEI-OC1 cells (Fig. 3A). To address the roles of individual MAPK pathways in HO-1 expression induced by phloretin, we examined the effects of a p38 inhibitor SB203580, a JNK inhibitor SP600125,

and an ERK inhibitor PD098059 on phloretin-induced expression of HO-1. Phloretin-induced HO-1 expression was directly related to the JNK pathway because the inhibitor SP600125 blocked the expression completely, whereas similar concentrations of PD98059 and SB203580 had no significant effect (Fig. 3B and C). These results indicated that kinases of the JNK pathway might be involved in the expression of HO-1 by phloretin.

The effects of phloretin on cisplatin-mediated action

We examined the effect of phloretin on cisplatin-induced apoptosis on HEI-OC1 cells. As shown in Figure 4A, incubation of the cells with 20 μM cisplatin for 36 h resulted in a 40% reduction of cell viability. However, pre-incubation of the cells with phloretin for 12 h diminished cisplatin-induced apoptosis in a dose-dependent manner (Fig. 4A). A TUNEL assay revealed that phloretin abolished the apoptotic response in cisplatin-treated cells (Fig. 4C). We also examined whether HO-1 expression was responsible for the protection afforded by phloretin against the apoptotic effect of cisplatin. The involvement of HO-1 in the protective effect of phloretin was confirmed using an inhibitor of HO activity, ZnPP IX. ZnPP IX blocked the protective effect of phloretin on cisplatin-induced apoptosis (Fig. 4B). These results demonstrated that the observed protective effect of phloretin on cisplatin-induced apoptosis was due to HO-1 expression. We also examined the effect of phloretin on the expression of Bcl-2 family proteins, including Bcl-2 and Bax, by cisplatin. Cells were treated with cisplatin for 36 h following pretreatment with phloretin for 12 h and then were lysated. The lysate was used to measure the expressions of Bcl-2 and Bax by Western blot analysis. Treatment of cells with cisplatin decreased the expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax. However, phloretin prevented the decrease in the expression of Bcl-2 while it markedly suppressed the increase of Bax expression in the cisplatin-treated cells (Fig. 5A). Next, we investigated the inhibitory effect of phloretin against the activation of caspase-8, -9, and -3 after cisplatin treatment. Cells

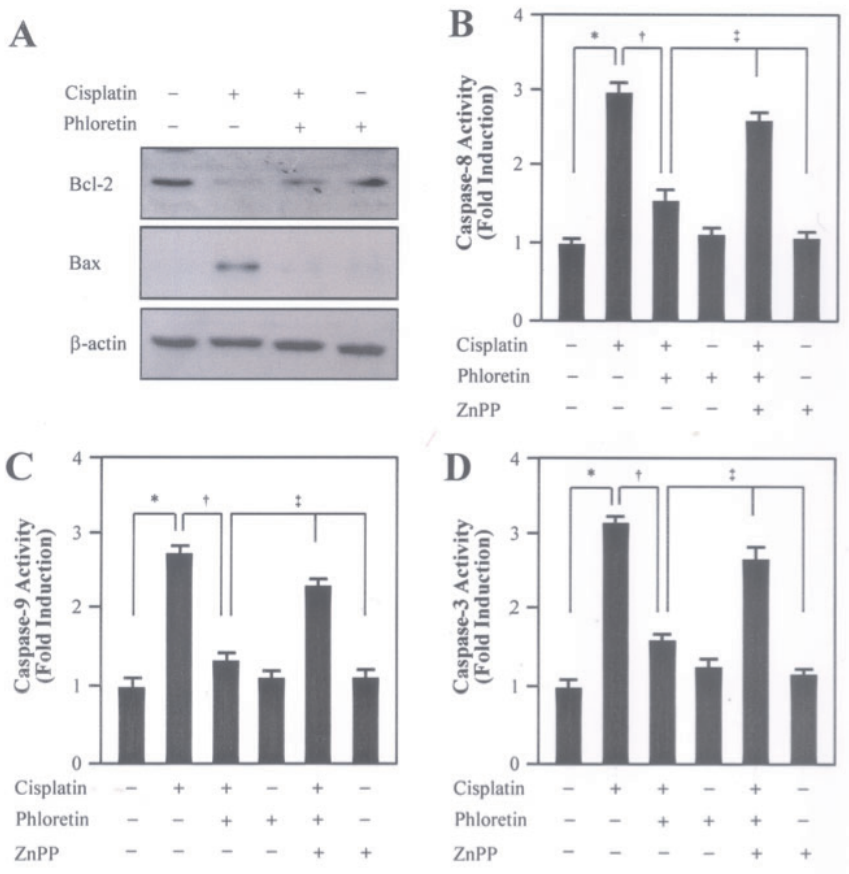

Fig. 5. Preventive effect of phloretin on the level of Bcl-2 family proteins and the activation of caspases by cisplatin in HECI-OC1 cells. (**A**) Cells were treated with 20 μM cisplatin for 30 h following pretreatment with 10 μM phloretin for 12 h. Cellular proteins were isolated, and Western blot analysis was then performed using specific antibodies for Bcl-2, and Bax. Data show the results of one of three independent experiments. (**B**–**D**) Cells were pretreated with 10 μM phloretin for 12 h in the absence or presence of 10 μM ZnPP and were then incubated with 20 μM cisplatin for 30 h. The activity of caspases was detected as described under "Materials and Methods". Data represent the means ± SD of three independent experiments. *, †, ‡ $p < 0.05$

were treated with cisplatin for 36 h and then were lysated. The lysate was used to measure the activation of caspase- 8, -9, and -3. As shown in Fig. 5B-D, the activation of caspase-8, -9, and -3 was significantly increased after cisplatin treatment. However, phloretin blocked the activation of caspase-8, -9, and -3 in cisplatin-treated cells.

Discussion

Recently, much attention has been focused on phytochemicals, as they can facilitate a number of antioxidative mechanisms, including HO-1, which show protection against oxidative cell damage [30]. In this study, we have provided evidences for the induction of HO-1 by phloretin through the Nrf2 and JNK pathways in HEI-OC1 cells. Phloretin protected the cells against cisplatin-induced apoptotic cell death *via* HO-1 induction. We also observed that exposure to phloretin diminished mitochondrial dysfunction and the activity of caspases.

The transcription factor Nrf2 plays an essential role in the expression of antioxidant enzymes and in the activation of other stress-inducible genes in response to oxidative stress [21]. As shown in Figure 2, the nuclear translocation of Nrf2 was essential for the induction of HO-1 expression by phloretin, consistent with the previous report that Nrf2 can bind to the ARE in the promoter regions of many stress-activated genes such as HO-1 [5]. In line with this finding, the translocation of Nrf2 into the nucleus following resveratrol treatment was associated with increases in its ARE-binding and transcriptional activity, as well as a marked increase in HO-1 expression [5, 18]. Our results suggest that Nrf2 may play a key role in phloretin-induced HO-1 expression. Transcriptional activation of HO-1 and other genes is mediated by a network of signaling pathways and by modulation of transcription factors. The role of MAPK pathways in the process of HO-1 expression has been demonstrated [6, 14, 26, 29]. In this study, we found that phloretin-induced HO-1 expression was directly related to the JNK pathway because the JNK inhibitor SP600125 blocked the expression completely, whereas inhibitors of p38 and ERK had no significant effect (Fig. 3). Consistent with our results, piperine and phorone promote a JNK-dependent induction of HO-1 expression [6, 28]. However, diallyl sulfide and arsenite promote an ERK-dependent induction of HO-1 expression [14, 18] and sulforaphane increases HO-1 expression through the activation of p38 [26]. One possible interpretation of these diverging observations may stem from the diverse assortment and intensity of the signaling pathways activated by different inducers in different cell types.

HO-1 has been recently recognized as an important cellular defense mechanism against various stresses [9, 28]. We found that the observed protective effect of phloretin on cisplatin-induced apoptotic cell death was due to HO-1 expression (Fig. 4). Although antioxidant activity and antiproliferative effects of phloretin have been reported [13, 31], our results provide the first evidence indicating that the induction of HO-1 by phloretin may serve as one of the important mechanisms of this protective effect. Previous studies revealed that cisplatin induced the apoptotic death of auditory cells through the generation of reactive oxygen species (ROS) and that HO-1 attenuated the cisplatin-induced apoptosis of auditory cells through downregulation of ROS generation [27, 34]. Indeed, antioxidants have demonstrated efficacy in the attenuation of noise-induced hearing loss, significantly protecting auditory outer hair cells [33]. Apoptosis is generally associated with caspase cascades and the Bcl-2 family of proteins [1, 19]. Cisplatin induced apoptotic cell death through activation of caspase-3, -8 and -9. In the present study, phloretin prevented the decrease in the expression of Bcl-2 protein, whereas it markedly suppressed the increase of Bax expression in the cisplatin-treated cells (Fig. 5A). Also, phloretin blocked the activation of caspase-8, -9, and -3 in cisplatin-treated cells (Fig. 5B-D). In addition, our previous studies showed that luteolin inhibited the activation of caspase-3 and the mitochondrial dysfunction caused by cisplatin [7]. Traditionally, cisplatin-induced ototoxicity appears to result from the destruction of outer hair cells in the organ of Corti as a result of apoptosis. A recent study showed that cisplatin-related apoptosis evokes an intrinsic proapoptotic signaling pathway within the rat's cochleae [16]. Another *in vivo* study showed that activation of caspase-9 and caspase-3, but not caspase-8, was detected after treatment with CDDP, and the cleavage of fodrin by activated caspase-3 was observed within damaged hair cells [35]. However, the present study and other *in vitro* models indicated the involvement of extrinsic as well intrinsic pathways [12].

In conclusion, the present results suggest that phloretin induces HO-1 expression *via* the Nrf2 and JNK

pathways, and HO-1 expression by phloretin could contribute to cellular defense mechanisms against cisplatin-induced apoptosis. Therefore, phloretin may be used to decrease the ototoxicity of cisplatin. Extensive further studies are ongoing to define the *in vivo* effect of phloretin on the systematic administration of cisplatin.

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