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*Research Paper*

# Kaempferol Suppresses Cisplatin-Induced Apoptosis Via Inductions of Heme Oxygenase-1 and Glutamate-Cysteine Ligase Catalytic Subunit in HEI-OC1 cells

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***Purpose.*** The present study was undertaken to elucidate the chemoprotective mechanism of kaempferol, which possesses anti-oxidative and anti-apoptotic properties.
***Methods.*** House Ear Institute-Organ of Corti 1 (HEI-OC1) cells were treated with kaempferol in the presence or absence of cisplatin. Cisplatin-induced oxidative stress was assessed by analysis of Comet assay, DNA-laddering assay and activation of caspases. Heme oxygenase-1 (HO-1), mitogen-activated protein kinase (MAPK) pathway and nuclear factor-E2-related factor 2 (Nrf2) were measured by Western blot analysis. Transfection of small interfering RNAs (siRNA), glutathione (GSH) assay and RT-PCR were performed in this study.
***Results.*** Kaempferol protected cells against cisplatin-induced apoptosis in a dose-dependent manner in HEI-OC1 cells. Kaempferol-induced HO-1 expression protected against cell death though the c-Jun N-terminal kinase (JNK) pathway and by the aid of Nrf2 translocation. Kaempferol increased the cellular level of GSH and the expression of GCLC time-dependently. siRNA GCLC blocked the increase of GSH level by kaempferol and the protective effect of kaempferol against cisplatin-induced cell death.
***Conclusion.*** The expression of HO-1 by kaempferol inhibits cisplatin-induced apoptosis in HEI-OC1 cells, and the mechanism of protective effect is also associated with its inductive effect of GCLC expression.

**KEY WORDS:** cisplatin; kaempferol; MAPK; Nrf2 (HO-1, GCLC); oxidative stress.

## INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II; CDDP) is an anticancer chemotherapeutic agent that is widely used to treat various cancers including osteosarcoma, hepatoblastoma, neuroblastoma, germ cell tumors, head and neck cancers and some central nervous system tumors (1). However, multiple toxicities, such as peripheral neuropathy, nephrotoxicity, nausea/vomiting and ototoxicity, have been reported (2,3). Ototoxic hearing loss has been described as being more severe and frequent in patients receiving higher and repeated doses of cisplatin, especially children (2,3). The direct cytotoxic mechanisms of cisplatin include the formation of reactive oxygen species (ROS), mitochondrial dysfunction and DNA damage, which seem to be significant causes of hair cell loss (4).

Transcription of many oxidative stress inducible genes, including glutathione (GSH) S-transferase, NAD(P)H: quinine oxidoreductase, catalytic subunit of glutamate-cysteine ligase (GCLC) and heme oxygenase (HO-1), are regulated in part through *cis*-acting sequences known as antioxidant response elements (AREs) (5,6). The ARE located in the 5-flanking region of many genes is essential for both detoxification and maintenance of cellular reducing potential (7) and is transcriptionally activated by binding the nuclear factor-E2-related factor2 (Nrf2) (8). Thus, a powerful cluster of protective genes can be coordinately up-regulated through the ARE to reduce the damage caused by harmful toxicants (7,8). HO-1 modulates various cellular functions in stress-related conditions, including cytokine production, cell proliferation and apoptosis, catalyzing the first and rate-limiting step of heme degradation (2,9). It is known that the up-regulation of HO-1 expression is induced by various injurious stimuli, such as ultraviolet irradiation, heat-shock, heavy metals, anoxia, hypoxia and hyperoxia (9). However, in recent years, numerous studies have suggested that the up-regulation of HO-1 expression with dietary compounds may play an important role in cellular defense mechanisms against oxidative insults (9,10).

In this study, we found that kaempferol, a natural compound isolated from green tea (11), broccoli, apple and berries (12), protected cells against cisplatin-induced apoptosis. The mechanism of the protective effect was investigated in House Ear Institute-Organ of Corti 1 (HEI-OC1) cells, which are auditory cells of mice. We demonstrated that kaempferol could induce the expressions of HO-1 and GCLC in HEI-OC1 cells. The c-Jun N-terminal kinase (JNK) path-

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way was involved in the induction of HO-1 by kaempferol and subsequently the signal from activated JNK translocated Nrf2 into nucleus. Kaempferol also increased the cellular level of GSH, and its protection of apoptotic cell death disappeared in the present of small interfering RNA (siRNA) GCLC. These results suggest a possible route for the relief of cisplatin-related hearing loss by dietary substances.

## MATERIALS AND METHODS

### Reagents

Kaempferol and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Zinc protoporphyrin IX (ZnPP IX) was from Porphyry Products (Logan, UT, USA). PD098059, SB203580, SP600125 and anti-HO-1 antibodies were obtained from Calbiochem (San Diego, CA, USA). Antibodies against Nrf2 (sc-722, Lot # L1905), Keap1 (sc-15246, Lot # F1308) and β-actins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-JNK, extracellular signal-regulated kinase (ERK) and p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Unless indicated otherwise, all other chemicals were obtained from Sigma-Aldrich.

### Cell Culture

The establishment and characterization of conditionally immortalized HEI-OC1 cells were done as previously described (13). HEI-OC1 cells have been recently established and characterized from long-term cultures of immortal-mouse cochlea. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL, Grand Island, 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 $5\times10^4$ cells/well. Cells were treated with cisplatin in the presence or absence of kaempferol, ZnPP or SP60025. Cell viability was detected by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay with the CellTiter96® aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. The absorbance was read at 490 nm on an enzyme-linked immunosorbent assay (ELISA) reader, and the percentage of cell survival was determined.

### Comet Assay

Cells were harvested and washed twice with ice-cold phosphate buffered saline (PBS). Generally, the slides were prepared one day before use. Seventy-five microliters of low melting point agarose (LMPA) (37˚C) mixed with 10,000 cells in a volume of 5–10 μl were added to the coated slides over an area of 24×50 mm. The cover slip was slid off, and 75 μl LMPA were added. The slides were put into cold, freshly made lysis solution, protected from light, and refrigerated for a minimum of 1 h. Subsequently, each slide was rinsed in Tris-HCl buffer (pH 7.4) to remove detergents and salts. Slides were subjected to electrophoresis for 20–60 min (25 V, 300 mA). Slides were placed on a drain tray and then dipped in neutralization buffer for at least 5 min two more times. Slides were exposed to cold 100% ethanol or cold 100% methanol, and flooded with 1×ethidium bromide stain for 5 min. Each slide was covered with a fresh cover slip and observed using a fluorescent microscope. Both photos and the Olive Tail Moments were recorded and measured by use of Komet 5.5 software (Kinetic Imaging Ltd., Liverpool, UK).

### Genomic DNA Isolation and DNA-Laddering Assay

Genomic DNA was isolated from cultured cells with the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA). Briefly, cells were lysed and treated with lysis buffer, followed by 1 h incubation with RNase A. The cell lysates were precipitated for proteins and spun at 15,000 rpm for 20 min. The supernatant was precipitated with isopropanol for isolation of DNA. After washing with 70% ethanol, DNA was hydrated and quantified. Equal amounts (10 μg) of DNA were electrophoresed on a 1.5% agarose gel (with incorporated ethidium bromide). The gel was then photographed under ultraviolet luminescence.

### Preparation of Cytosolic and Nuclear Extracts

Cytosolic and nuclear fractions were prepared as previously described (14). Briefly, cells were washed three times with cold PBS and centrifuged at 10,000 rpm for 10 min. The pellet was carefully suspended in 50 μl lysis buffer (20 mM HEPES, pH 7.0, 0.15 mM EGTA, 10 mM KCl, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM Na3VO4) and incubated on ice for 15 min. At the end of this incubation, 3 μl of 10% Nonidet-40 were added, and the tubes were vortexed for 10 s. The homogenate was then centrifuged at 13,000 rpm for 20 min, and the nuclear pellet was washed in five pellet volumes of cold PBS. After centrifugation at 13,000 rpm for 20 min, nuclei were suspended in 30 μl of hypertonic cold buffer containing 10 mM HEPES, pH 8.0, 25% glycerol, 0.4 M NaCl, 0.1 mM EDTA, 1 μg/ml leupeptin 1 mM PMSF, 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM Na3VO4 and incubated for 30 min at 4°C on a rotating wheel. Nuclear debris was removed by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and submitted to Western blot using anti-Nrf2 and anti-Lamin B antibodies.

### Transient Transfection

A day before transfection, cells were subcultured at a density of $1\times10^6$ cells in 60 mm-diameter dishes to attain 70–80% confluence. The cells were transiently transfected using lipofectamine with the plasmid containing dominant-negative (DN) of Nrf2 or JNK DN, according to the instructions provided by the manufacturer (GIBCO-BRL, Grand Island, NY, USA). After overnight transfection, cells were treated

with 10 μM kaempferol for 18 h, and protein expression was ascertained by Western blot.

### Western Blot Analysis

Briefly, cells were harvested, washed twice with ice-cold PBS and suspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture comprised of 0.1 mM PMSF, 5μg/ml aprotinin, 5μg/ml pepstatin A and 1μg/ml chymostatin. Protein concentration was determined with a commercial Lowry protein assay kit. Proteins were separated by 12% SDS-PAGE, and the separated proteins were electrically transferred to a nitrocellulose sheet. Each membrane was sequentially incubated for 1 h in the presence of primary and secondary antibodies. The protein bands were visualized using chemiluminescent reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

### Caspase Activity Assay

The activities of caspase-3, -8 and -9 were analyzed using the appropriate caspase assay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted as described previously (15). PCR conditions for GCLC and β-actin were as follows: 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. The primer pairs were as follows: GCLC, (forward, 5′-ACAAGCACCCCC GCTTCGGT-3′ and reverse, 5′-CTCCAGGCCTCTCTCC TCCC-3′; β-actin, (forward, 5′-CCTTCTACAATGAGC-3′ and reverse, 5′-ACGTCACACTTCATG-3′). Amplification products were resolved by 1.2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

### GSH Determination

GSH levels in HEI-OC1 cells were assessed using a glutathione assay kit (Calbiochem, San Diego, CA, USA). After exposure to kaempferol for the indicated times, cells were harvested and homogenized in 5% metaphosphoric acid working solution. After centrifugation, 50μl of R1 solution (chromogenic reagent in HCl) was added to 900μl final volume (Solution 3 and sample), followed by gentle vortexing. Subsequently, following the addition of 50μl R2 solution

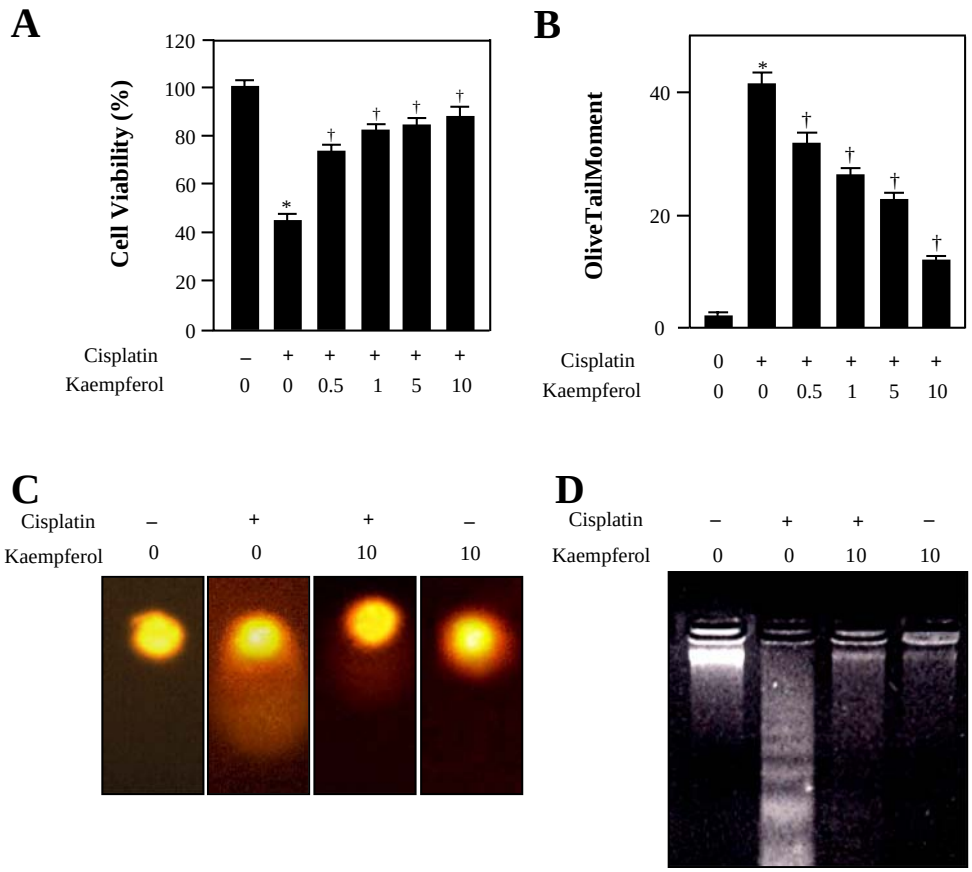


**Fig. 1.** Protective effect of kaempferol on cisplatin-induced toxicities in HEI-OC1 cells. (A and B) Cells were pretreated with the indicated doses of kaempferol for 18 h and were then incubated with 20μM cisplatin for 30 h. (**A**) Cell viability was measured by a MTS assay. (**B**) Olive Tail Moments were recorded and measured by the Comet assay using Komet 5.5 software. (C and D) Cells were pretreated with or without 10μM kaempferol for 18 h, and then incubated with 20μM cisplatin for 30 h. (**C**) DNA damage was ascertained by the Comet assay (**D**) Genomic DNA was isolated from cells and separated by 1.5% agarose gel electrophoresis. Data represent the mean ± S.D. of three independent experiments. * $P<0.05$ *versus* control; †$P<0.05$ *versus* cisplatin.

(30% NaOH), the mixture was incubated at 25°C for 10 min in the dark. The final absorbance of the mixture was read at 400 nm. The protein concentration was determined using a Lowry protein assay kit.

### siRNA Transfection

Predesigned siRNA GCLC were purchased from Invitrogen (Carlsbad, CA, USA). The siRNA GCLC construct was forward, 5′-UUUCUUGUUAGAGUACCGAAG CGGG-3′ and reverse, 5′-CCCGCUUCGGUACUCUAACAAGAAA-3′. The siRNA HO-1 and control siRNA instructions were provided by the manufacturer (Santa Cruz Biotechnology). Cells were transfected with either total 40 nM negative control siRNA or negative control siRNA containing siRNA HO-1 or siRNA GCLC for 12 h using Opti-MEM®I reduced serum medium (GIBCO BRL, Grand Island, NY, USA) utilized lipofectamine according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA), prior to recovery in fresh DMEM. Cells were further treated with cisplatin and kaempferol. Samples were then prepared and analyzed for viability or GSH assay.

### Statistical Analysis

Statistical analysis was done using one-way ANOVA, and all values are expressed as mean ± S.D. The differences between groups were considered to be significant at $P<0.05$.

## RESULTS

### Kaempferol Protects HEI-OC1 Cells Against Cisplatin-Induced Apoptosis

We examined the effect of kaempferol on cisplatin-induced cell death in HEI-OC1 cells. As shown in Fig. 1A, incubation of cells with 20 μM cisplatin for 30 h resulted in a 53% reduction of cell viability. However, pretreatment of cells with kaempferol for 18 h diminished cisplatin-induced cell death in a dose-dependent manner, and the protective effect of kaempferol was pronounced at a concentration of 10 μM. Comet assay was conducted to assess whether kaempferol could protect cells against cisplatin-induced DNA oxidative damage. Significant increases in the Olive tail moment of cisplatin-treated cells were

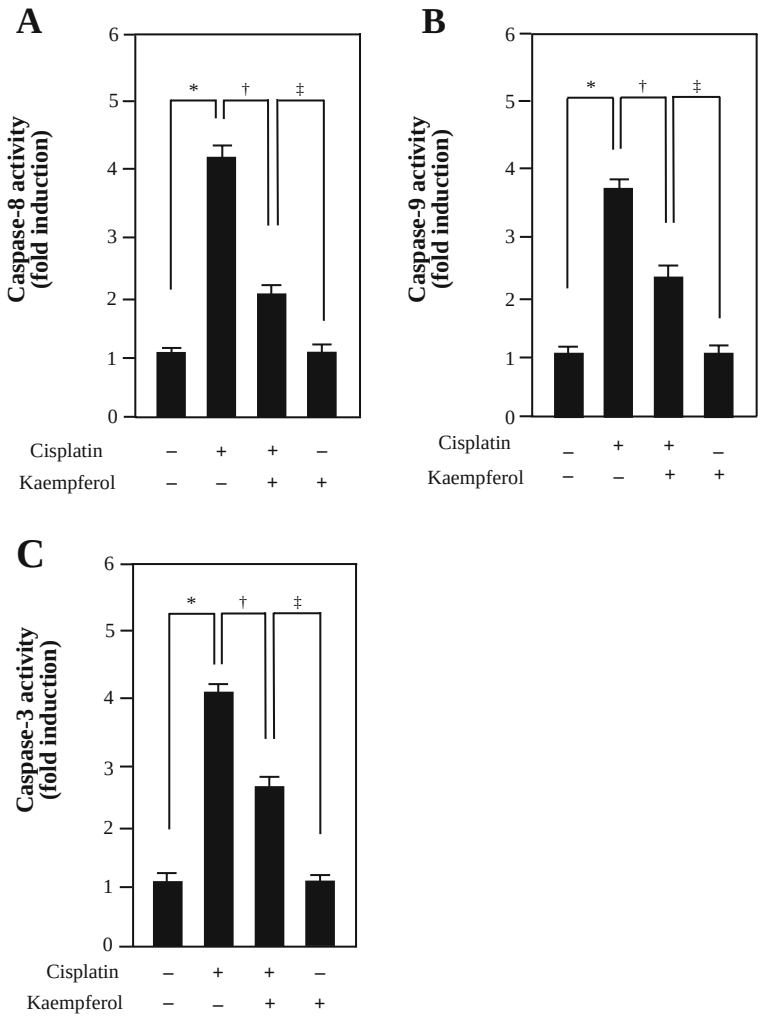


**Fig. 2.** Kaempferol down-regulates the activities of caspase-3, -8 and -9 in HEI-OC1 cells. (**A**, **B** and **C**) Cells were pretreated with 10 μM kaempferol for 18 h and incubated with 20 μM cisplatin for 30 h. The activities of caspase-3, -8 and -9 were analyzed as described under Materials and Methods. Data represent the mean ± S. D. of three independent experiments. *, †, ‡ $P<0.05$.

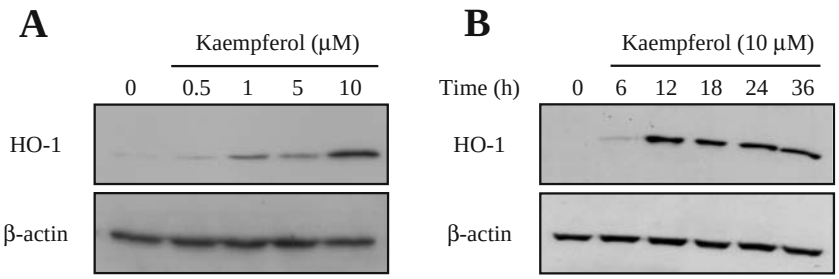


**Fig. 3.** Induction of HO-1 expression by kaempferol in HEI-OC1 cells. (**A**) Cells were incubated with various concentrations of kaempferol for 18 h, and level of HO-1 was measured. (**B**) Cells were treated with 10 μM kaempferol for the indicated time, and then the HO-1 level was measured. Cells were lysed, and Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Experiments were repeated three times with similar results.

evident (Fig. 1B and 1C). DNA damage was abrogated by kaempferol pretreatment in a dose-dependent manner up to a concentration of 10 μM. As shown in Fig. 1D, cells which were treated with cisplatin only showed apparent DNA laddering, while the laddering was blocked by pretreatment with kaempferol. The activation of caspase cascades is a critical component of the initiation of apoptosis in many biological systems (16). To address if the anti-apoptotic effect of kaempferol was coincident with reduced caspase activity, cells were treated with cisplatin for 30 h, and the activities of caspases-3, -8 and -9 in cell lysates was determined. Kaempferol evidently lowered all three caspases (Fig. 2). These results suggested that the apoptosis occurring in cisplatin-induced cells could be blocked at least partially by kaempferol.

### Kaempferol Induces HO-1 Expression by the Aid of Nrf2 in HEI-OC1 Cells

The effect of kaempferol on HO-1 expression in HEI-OC1 cells was determined. The cells were treated with 0.5–10 μM of kaempferol for 18 h. Kaempferol affected HO-1 expression in a dose-dependent manner (Fig. 3A). Treatment with 10 μM kaempferol produced an increase in the HO-1 level, which was slight at 6 h post-treatment and increased thereafter, reaching a

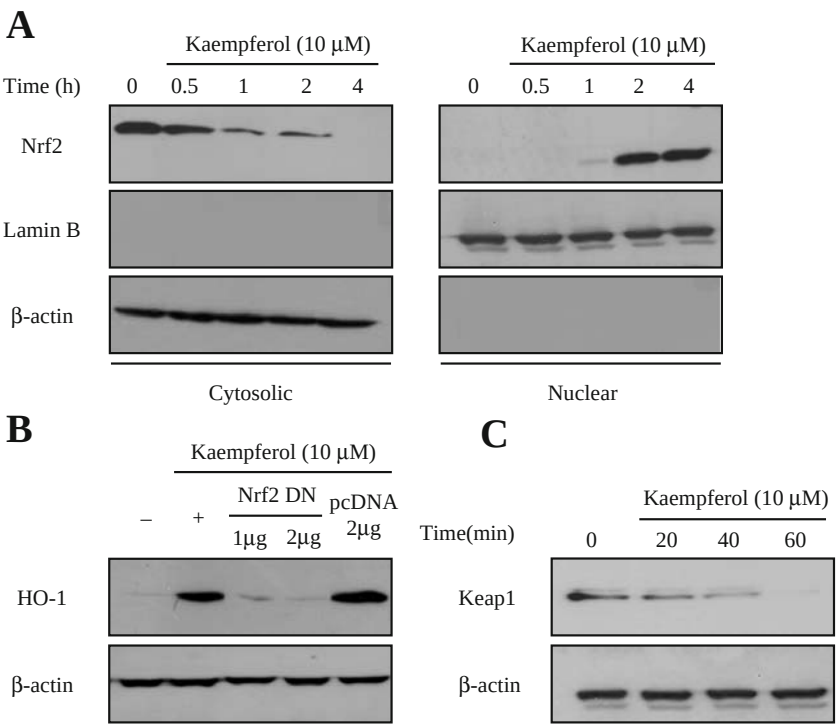


**Fig. 4.** Involvement of Nrf2 in kaempferol-induced HO-1 expression. (**A**) HEI-OC1 cells were treated with 10 μM kaempferol for various times. After harvest, both the cytosolic and nuclear fractions were isolated. Nrf2 protein of each fraction was detected by Western blotting. (**B**) HEI-OC1 cells were transfected transiently with the indicated amount of Nrf2 DN plasmid. After 18 h of incubation, cells were incubated with 10 μM kaempferol for 18 h. The levels of HO-1 and β-actin were detected by Western blotting. (**C**) HEI-OC1 cells were treated with 10 μM kaempferol and then harvested at the times indicated in the figure. The levels of Keap1 in the cell lysates were detected by Western blotting. Experiments were repeated three times with similar results.

plateau at 12 h (Fig. 3B). Since nuclear translocation of activated Nrf2 is an important upstream step in the mechanism of HO-1 expression (17), we investigated whether treatment with kaempferol could induce Nrf2 translocation into the nuclei of HEI-OC1 cells. Cells were treated with 10 μM kaempferol for 0.5, 1, 2 and 4 h, and the level of Nrf2 protein was determined in the cytosol and nuclei by Western blotting. Kaempferol decreased the level of Nrf2 in cytosol but induced the accumulation of Nrf2 in nuclei (Fig. 4A). Cells were transfected with a DN plasmid of Nrf2 and then were treated with kaempferol. As shown in Fig. 4B, Nrf2 DN substantially attenuated kaempferol-mediated induction of HO-1 in a dose-dependent manner. This result demonstrated that the nuclear translocation of Nrf2 was essential in the process of HO-1 expression by kaempferol. It has been reported that Keap1 binds with Nrf2 in the cytoplasm and suppresses Nrf2 transcriptional activity. Removing of Keap 1 from Nrf2 translocates Nrf2 to the nuclei, and it potentiates the ARE-related gene expression (18). Therefore, we detected the level of Keap1 in kaempferol-treated cells. Figure 4C showed that most of the level of Keap1 in the cytosol was reduced within 40 min after kaempferol treatment.

### Kaempferol-Induced HO-1 Expression is Mediated by JNK Pathway

We investigated whether kaempferol could induce HO-1 expression through a specific mitogen-activated protein kinase (MAPK) pathway. The results showed that kaempferol activated the JNK pathway only among the MAPK pathways (Fig. 5A). To confirm the roles of the individual MAPK pathways in HO-1 expression induced by kaempferol, the effects of PD098059, SB203580 and SP600125 on kaempferol-induced HO-1 expression were assessed. HO-1 expression was completely blocked by SP600125, a specific inhibitor of JNK, while similar concentrations of SB203580 and PD098059 had no significant effect (Fig. 5B). To further prove the role of the JNK pathway in HO-1 expression by kaempferol, JNK DN plasmid was transfected into the cells, and it diminished the expression of HO-1 in HEI-OC1 in a dose-dependent manner (Fig. 5C). These results indicated that the JNK pathway was involved in kaempferol-induced HO-1 expression.

### JNK Pathway Induces the Nuclear Translocation of Nrf2

Recently, a few of studies have demonstrated that MAPKs signaling pathway is closely related to the activation and nuclear translocation of Nrf2 (19–21). Therefore, to further elucidate the mechanisms by which nuclear translocation of Nrf2 could be involved in kaempferol-induced HO-1 expression, we tested Nrf2 nuclear translocation occurring downstream of JNK pathway. Cells were transfected with the indicated amount of JNK DN plasmid and then incubated with 10 μM kaempferol. JNK DN blocked the nuclear translocation of Nrf2 in a dose-dependent way (Fig. 6A), and pretreatment of SP600025 visibly attenuated kaempferol-mediated nuclear translocation of Nrf2 (Fig. 6B). As shown in Fig. 4C, the level of Keap1 in the cytosol was invisible completely at 1 h after kaempferol treatment. In parallel with this observation, when cells were pretreated with the indicated amount of JNK DN plasmid for 1 h, the level of Keap1 in the lysate did not disappear in a dose-dependent manner (Fig. 6C). The results showed that the effect of kaempferol on Keap 1 was blocked by JNK DN plasmid completely.

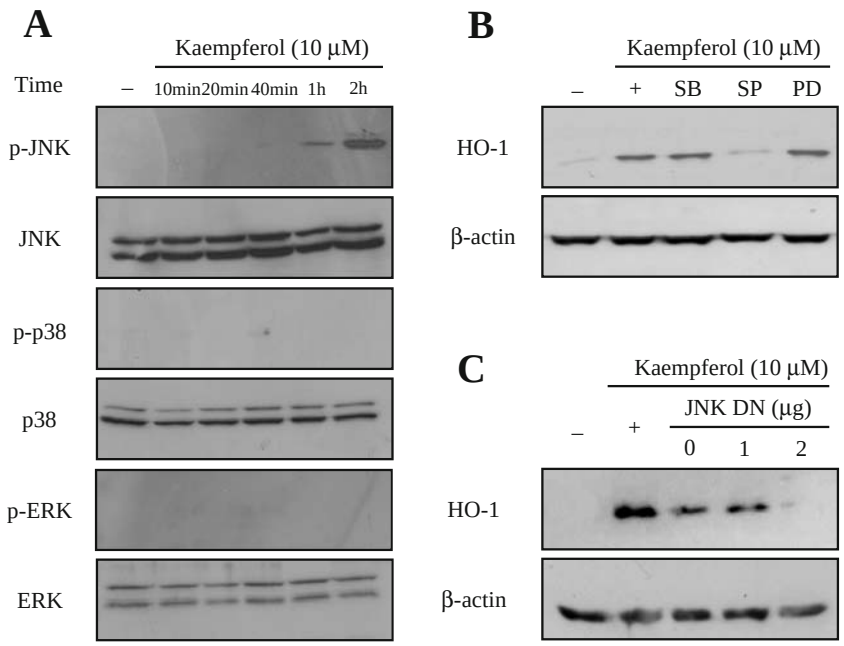


**Fig. 5.** JNK signal pathway is linked to kaempferol-induced HO-1 expression. (**A**) Cells were treated with 10 μM kaempferol for 10 min to 2 h, and the activations of MAPK pathways were determined by Western blot analysis using anti-phospho JNK, ERK or p38 anti-bodies. (**B**) Cells were pretreated with 20 μM SB203580 (SB), 20 μM SP600125 (SP) or 20 μM PD098059 (PD), and were then incubated in the presence of 10 μM kaempferol for 18 h. (**C**) Cells were transfected with the indicated amounts of JNK DN plasmid. After 18 h of incubation, the cells were stimulated with 10 μM kaempferol for 18 h. Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Experiments were repeated three times with similar results.

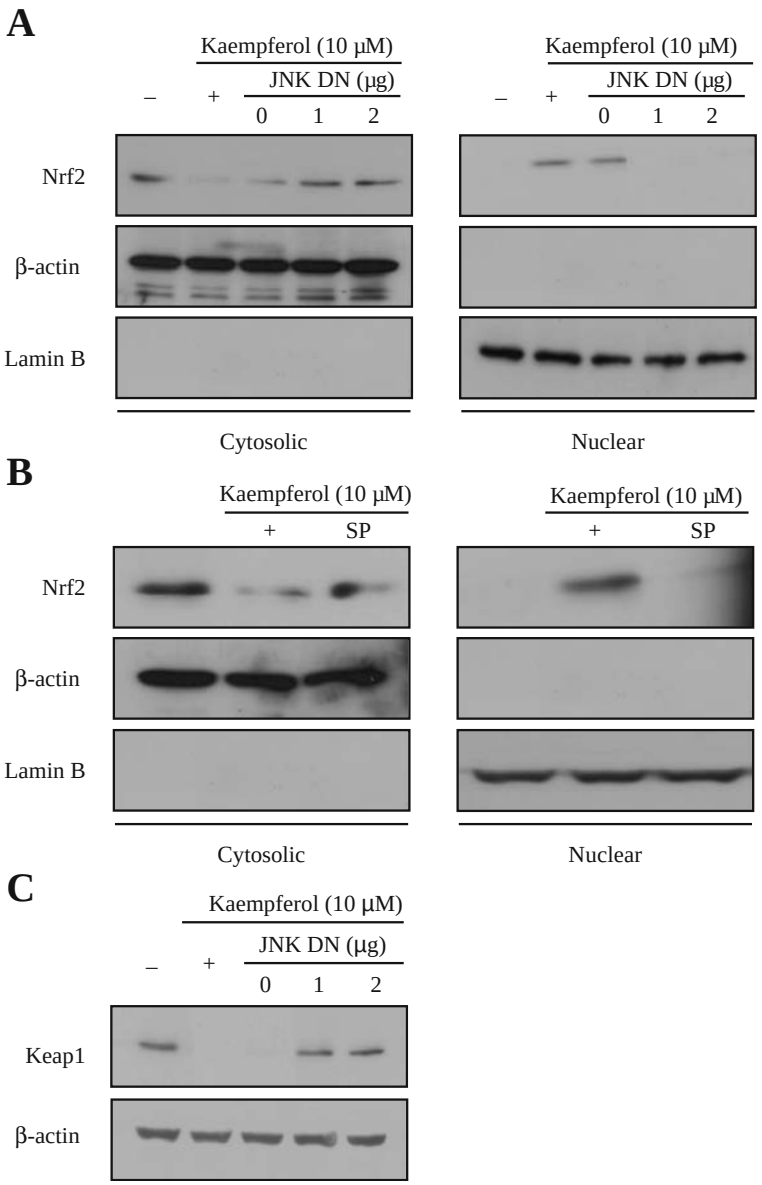


**Fig. 6.** The activation of JNK pathway by kaempferol translocates Nrf2 into nucleus. (**A**) HEI-OC1 cells were transfected with the indicated amount of JNK DN plasmid. After 18 h of incubation, cells were incubated with 10 μM kaempferol for 18 h, and then the level of Nrf2 was measured in both cytosolic and nuclear fractions. (**B**) Cells were pretreated with SP600125 for 30 min, followed by incubation of 10 μM kaempferol for 18 h, and then the level of Nrf2 was measured in both cytosolic and nuclear fractions. (**C**) HEI-OC1 cells were transfected with the indicated amount of JNK DN plasmid. After 18 h of incubation, cells were incubated with 10 μM kaempferol for 1 h, and then the level of Keap 1 was measured in cell lysate. Western blot analysis was performed using specific antibodies for Nrf2, Keap 1, Lamin B and β-actin. Experiments were repeated three times with similar results.

### Kaempferol Increases the Cellular Level of GSH and Protects Cells from Cisplatin-Induced Cell Death by Increasing the Expressions of HO-1 and GCLC

HEI-OC1 cells were treated with 10 μM kaempferol, and the expression of GCLC was examined with RT-PCR. The GCLC expression by kaempferol was readily detected within 2 h treatment and increased time-dependently up to 24 h (Fig. 7A). We next tested whether JNK pathway and nuclear translocation of Nrf2 also play roles in the expression of GCLC gene. As shown in Fig. 7B and C, the JNK DN and Nrf2 DN plasmid could reduce the expression of GCLC in a dose-dependent fashion. Compared with the expression of GCLC, the GSH level was decreased up to 4 h following kaempferol treatment, and it was gradually increased after 4 h and reached 1.2-fold of the control at 24 h. However,

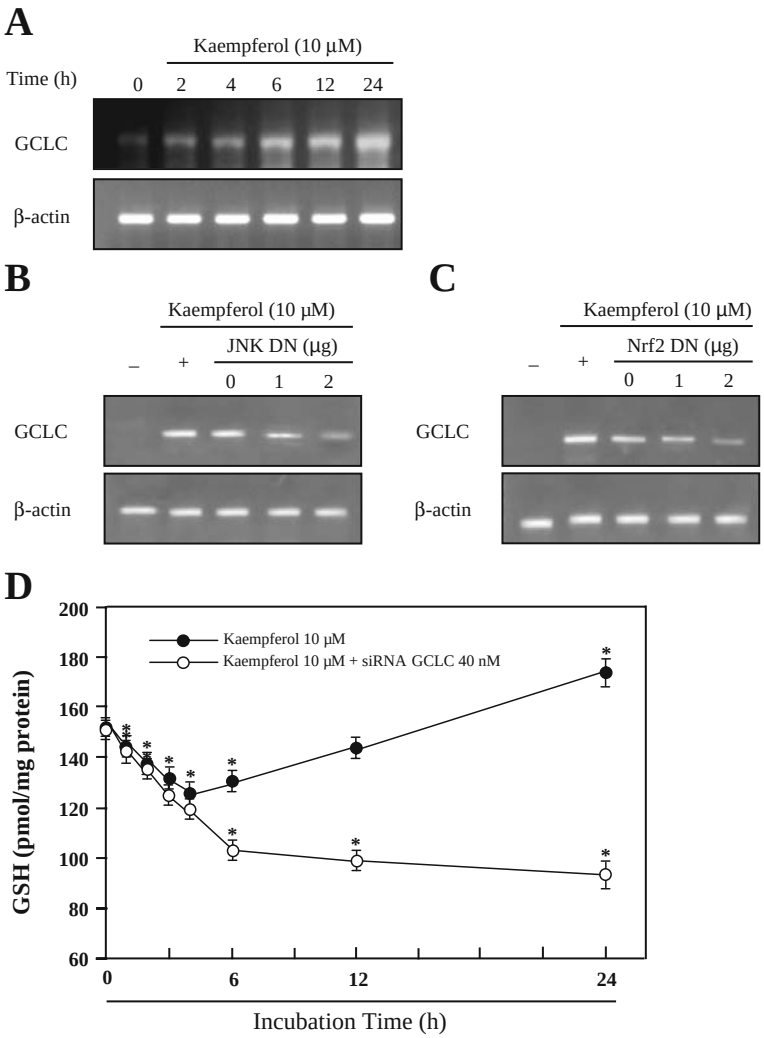


**Fig. 7.** Effect of kaempferol on the expression of GCLC and level of GSH in HEI-OC1 cells. (A) Cells were treated with 10μM kaempferol for the indicated time, and GCLC expression was analyzed by RT-PCR. (**B** and **C**) HEI-OC1 cells were transfected with the indicated amount of JNK DN and Nrf2 DN plasmid for 18 h to be followed by incubation of 10μM kaempferol. GCLC expression was analyzed by RT-PCR. (**D**) Cells were transfected with 40 nmol/ml siRNA control or siRNA GCLC. After 18 h of incubation, cells were treated with kaempferol for the times indicated in the figure, and then the detection of cellular GSH level was detected. The experiments were repeated three times with similar results. Data represent the mean ± S.D. of three independent experiments. * $P<0.05$ *versus* control.

when cells were transfected with siRNA GCLC before kaempferol incubation, the cellular level of GSH was decreased gradually from beginning and then reached 61% of the control at 24 h (Fig. 7D). Since kaempferol showed inductive effects on HO-1 and GCLC expressions (Figs. 3 and 7A), we examined whether the expressions were related to the protective effect of kaempferol against cisplatin-induced cell death. The involvement of HO-1 in the protective effect of kaempferol was investigated using an inhibitor of HO activity, ZnPP IX and SP600125, which blocked the expression of HO-1 by kaempferol (Fig. 5B). Pretreatment with ZnPP IX or SP600125 completely abolished the protective effect of kaempferol (Fig. 8A). These results demonstrated that the observed protective effect of kaempferol against cisplatin-induced cell death was due to its inductive effect on HO-1 expression. Likewise, the treatment with N-acetylcysteine (NAC), which increases the endogenous GSH level (22), also protected cells from cisplatin-induced cell death. To further verify the specific involvement of HO-1 and GCLC, cells were transfected with HO-1 and GCLC-specific siRNA. As shown in Fig. 8B, the cytoprotection of kaempferol against cisplatin was abolished completely by the transfection with both siRNAs.

## DISCUSSION

In the present study, we examined whether kaempferol, one of the most commonly found dietary flavonoids (23), could overcome the oxidative damage induced by cisplatin in HEI-OC1 cells. Cisplatin-induced apoptotic death through activation

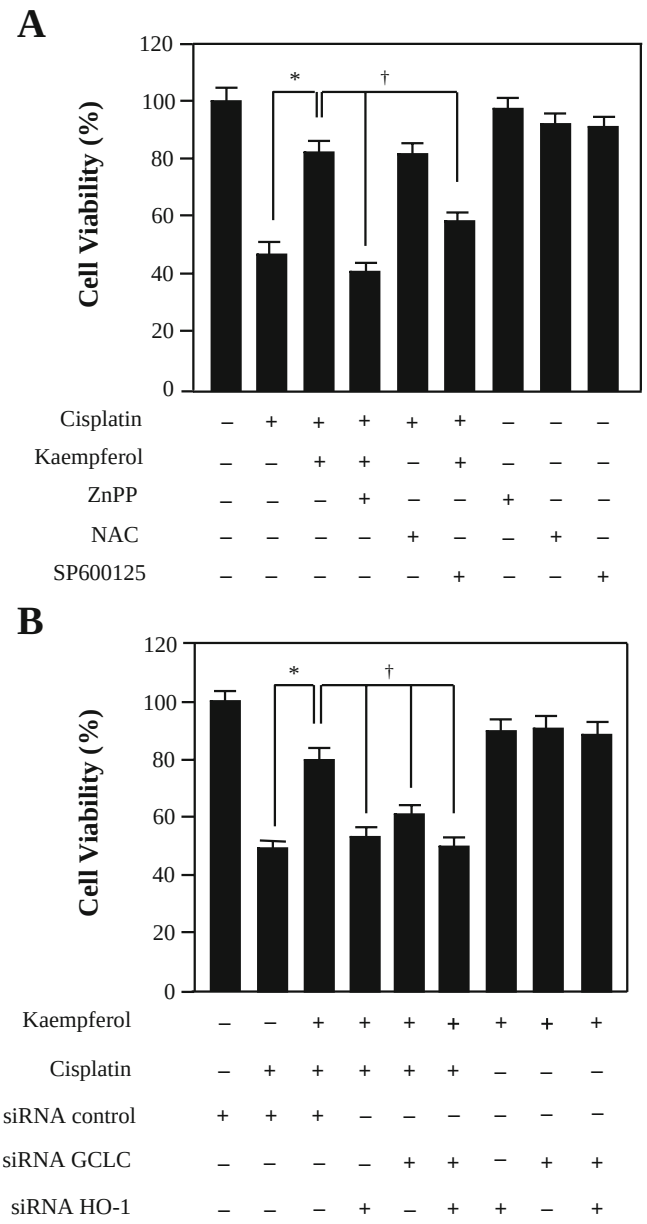


**Fig. 8.** Modulation of the protective effect of kaempferol on cisplatin-induced cell death. (**A**) Cells were pretreated with 10 μM kaempferol or 15 mM NAC for 18 h in the absence or presence of 10 μM Znpp and 20 μM SP600125 for 30 min. Then cells were incubated with 20 μM cisplatin for 30 h. (**B**) Cells were transfected with either 40 nM siRNAs of control, HO-1 or GCLC, and then were incubated with 10 μM kaempferol for 18 h, followed by cisplatin treatment for additional 30 h. Cell viability was measured using the MTS assay. Data represents the mean ± SD of three independent experiments. *, †$P<0.05$.

of caspase-3, -8 and -9 activities. However, exposure to kaempferol down-regulated activities of the caspases and protected the apoptotic death. There are some studies on the biological properties of kaempferol, especially its anti-apoptotic effect. It has been reported that kaempferol selectively reduces the apoptotic effect of 7β-hydroxycholesterol in rat vascular smooth muscle cells (23) and plays a considerable role against oxidative stress and apoptosis in rat H4IIE cells (24). Furthermore, kaempferol also efficiently blocks cerebellar granule cell death through low $K^+$-induced apoptosis (25). However, high concentration of kaempferol, usually over 30 μM, induces growth inhibition and apoptotic cell death by generation of ROS in some malignant cell lines (26,27). Cisplatin is used extensively as a chemotherapeutic agent in the treatment of various solid tumors (1). However, cisplatin has several side effects, including ototoxicity and nephrotoxicity, which greatly limit its utility and therapeutic profile (28). Recent research clarifies that such side effects are proved to be driven by direct cytotoxic mechanism, including DNA damage, mitochondrial dysfunction and the formation of ROS (4). Some free radical scavengers and antioxidants play pivotal roles in diminution of cisplatin-induced oxidative stress followed by apoptotic cell death (29). Herbal extracts, such as ginger and roselle, have been found to counteract cisplatin-induced cell death in rat testis (24). Epicatechin can also inhibit cisplatin-induced apoptosis and intracellular ROS generation (30). In addition, the activity of HO-1 can suppress the apoptotic effect of cisplatin (31,32).

To date, there is scant evidence that kaempferol can induce HO-1 expression. In the sole paper showing kaempferol-induced HO-1 expression, the authors did not report any dose-dependence and did not conduct any mechanistic studies on the expression (33). Thus, presently, it was a priority to investigate whether kaempferol was able to induce the expression of HO-1. Furthermore, the mechanistic pathway of the expression and the physiological role of the expression including GCLC were demonstrated. We found that kaempferol can potently prevent HEI-OC1 cell from cisplatin-induced apoptosis via up-regulation of HO-1 in time- and dose-dependent manners *in vitro*, producing a marked effect with 10 μM (Fig. 3).

The induction of HO-1 could suppress the apoptotic effect of cisplatin. The role of the oxidative stress in cisplatin-induced ototoxicity and nephrotoxicity is additionally supported by the protective effect of several free radical scavengers and antioxidants (34). HO-1 expression is correlated with the treatment of several flavonoids (35). Baicalein is able to induce HO-1 via ROS-dependent ERK activation, and the inductive effect indeed plays an important role in the anti-apoptotic effect of baicalein in RAW264.7 cells (36). Quercetin exerts potent hepatoprotective effects (35) that are associated with HO-1 induction. Similar up-regulation of HO-1 production occurs in the presence of curcumin (37), chalcone (38) and naringenin-7-O-glucoside (39). In a previous study, we also found that piperine protects cells against cisplatin-induced apoptosis by up-regulating the expression of anti-oxidative genes through the Nrf2 transcription factor pathway (31).

The cellular defense response to chemical or oxidative stress is characterized by a coordinated induction of phase II drug-metabolizing enzymes and GSH synthesis, which protects cells through the elimination of electrophiles. The transcription factor Nrf2 plays an essential role in the induction (40). As shown in Fig. 4, the nuclear translocation of Nrf2 was observed to be essential for the induction of HO-1 expression by kaempferol, 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 and GCLC (17). To investigate whether the anti-apoptotic effect of kaempferol was associated with its inductive effect on ARE-related gene expression, pre-treatment with siRNAs of HO-1 and GCLC was carried out. GCLC siRNA could block the

protective effect of kaempferol similarly to that observed with HO-1 siRNA (Fig. 8B). These results suggest that kaempferol is capable of obviating cisplatin-induced damage via expressions of HO-1 and GCLC. Although GCLC is an ARE-related gene, some inducers of ARE-related genes decrease intracellular GSH level at early stage of treatment (41,42). In response to this depletion, ARE-related genes can be induced very strongly by such inducers as a cell survival pathway (42,43). In this study, the cellular level of GSH was reduced when cells were exposed to 10μM kaempferol for up to 4 h, after which the GSH level increased gradually and reached 1.2-fold at 24 h (Fig. 7D). Fluctuating GSH levels have also been reported upon treatment with 3-morpholinosydnonimine hydrochloride, which can induce the expressions of HO-1 and GCLC in PC12 cells (43). However, the GSH level is restored to its basal level after the initial transient decreasing. Interestingly, acrolein treatment can rapidly reduce cellular GSH levels in a dose-dependent fashion, but only the lowest tested concentration could increase GSH level after 6 h in endothelial cells (44).

Transcriptional activation of HO-1 and other genes is mediated by a network of signaling pathways and by modulation of transcription factors. MAPKs regulate a number of cellular processes, such as cell growth, differentiation, stress responses and apoptosis (9). Oxidative agents, including heme, hyperoxia and ROS, induce HO-1 gene expression through activation of MAPKs (45). The role of MAPK pathways in the process of HO-1 expression has been demonstrated (9,46). In this study, kaempferol-induced HO-1 expression was directly related to the JNK pathway because a JNK inhibitor SP600125 and a JNK DN plasmid affected the kaempferol-induced change in the HO-1 protein level, while each inhibitor of ERK and p38 pathways did not (Fig. 5). The signal from the JNK pathway by kaempferol may transfer to Nrf2 because disturbance of JNK pathway by SP600125 and JNK DN kept Nrf2 in the cytosol and decreased the protective effect of kaempferol on cisplatin-induced cell death (Figs. 6 and 8A). In line with our results, GSH depletion and arsenite exposure promote a JNK-dependent induction of HO-1 expression (45). On the other hand, sulforaphane induces HO-1 expression via p38 pathways (10). Arsenite induces HO-1 expression via the ERK and p38 pathways in chicken hepatoma cells (47), but inhibition of p38 has no effect on cadmium- and hemin-dependent HO-1 expression in HeLa cells (10). Luteolin activates the ERK pathway (32), while piperine activated the JNK pathway (31).

In conclusion, kaempferol can suppress cisplatin-induced oxidative stress and apoptosis in HEI-OC1 cells by its induction of HO-1 and GCLC. The HO-1 up-regulation by kaempferol is performed via a special MAPK signaling pathway, the JNK pathway, which mediates Nrf2 translocation into the nucleus. In addition, kaempferol-mediated induction of GSH is also involved with the protective effect on cisplatin-induced cell death. Antioxidant compounds which react with oxidants and radicals directly play important roles in resistance to aging (48) and noise-induced hearing loss (2,48), but those compounds do not persist in cellular systems. However, considering that kaempferol has an intense effect on increasing antioxidant defense potential (ARE-related genes) of cells and exists in large quantities in various vegetables and fruits, the current study may offer a possible way to decrease hearing losses by aging, noise and/or cisplatin.

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