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Baicalein inhibition of hydrogen peroxide-induced apoptosis via ROS-dependent heme oxygenase 1 gene expression

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Abstract

In the present study, baicalein (BE) but not its glycoside, baicalin (BI), induced heme oxygenase-1 (HO-1) gene expression at both the mRNA and protein levels, and the BE-induced HO-1 protein was blocked by adding cycloheximide (CHX) or actinomycin D (Act D). Activation of ERK, but not JNK or p38, proteins via induction of phosphorylation in accordance with increasing intracellular peroxide levels was detected in BE-treated RAW264.7 macrophages. The addition of the ERK inhibitor, PD98059, (but not the p38 inhibitor, SB203580, or the JNK inhibitor, SP600125) and the chemical antioxidant, N-acetyl cysteine (NAC), significantly reduced BE-induced HO-1 protein expression by respectively blocking ERK protein phosphorylation and intracellular peroxide production. Additionally, BE but not BI effectively protected RAW264.7 cells from hydrogen peroxide (H_2O_2)-induced cytotoxicity, and the preventive effect was attenuated by the addition of the HO inhibitor, SnPP, and the ERK inhibitor, PD98059. H_2O_2-induced apoptotic events including hypodiploid cells, DNA fragmentation, activation of caspase 3 enzyme activity, and a loss in the mitochondrial membrane potential with the concomitant release of cytochrome *c* from mitochondria to the cytosol were suppressed by the addition of BE but not BI. Blocking HO-1 protein expression by the HO-1 antisense oligonucleotide attenuated the protective effect of BE against H_2O_2-induced apoptosis by suppressing HO-1 gene expression in macrophages. Overexpression of the HO-1 protein inhibited H_2O_2-induced apoptotic events such as DNA fragmentation and hypodiploid cells by reducing intracellular peroxide production induced by H_2O_2, compared with those events in neo-control (neo-RAW264.7) cells. In addition, CO, but not bilirubin and biliverdin, addition inhibits H_2O_2-induced cytotoxicity in macrophages. It suggests that CO can be responsible for the protective effect associated with HO-1 overexpression. The notion of induction of HO-1 gene expression through a ROS-dependent manner suppressing H_2O_2-induced cell death is identified in the present study.

Keywords: Heme oxygenase 1; Flavonoids; ROS; Apoptosis; ERKs; CO

1. Introduction

Flavonoids are phenolic compounds and exist widely in plants, fruits, and Chinese herbal medicine. In the past decade, the antioxidant activities of flavonoids have been given much attention due to many flavonoids having been found to possess better antioxidant activities than vitamins C and E. In addition to antioxidation, several beneficial effects including antitumor, anti-inflammatory and neuronal protective properties have also been identified [1–3]. Baicalein (BE), a major component of

Abbreviations: BE, baicalein; BI, baicalin; ERK, extracellular regulated kinases; JNK, c-Jun N-terminal kinases; HO-1, heme oxygenase 1; NAC, N-acetyl cysteine; CHX, cycloheximide; Act D, actinomycin D; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); DCHF-DA, 2′,7′-dichlorodihydrofluorescein-diacetate; PI, propidium iodine; DiOC6(3), NBT, 3,3′-dihexyloxacarbocyanine iodide nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; SnPP, tin protoporphyrin; LDH, lactate dehydrogenase; CO, carbon monoxide

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Scutellaria baicalensis, has been shown to be a lipoxygenase inhibitor, and it induces apoptosis in several cancer cells such as breast carcinoma cells, colon carcinoma cells, and leukemia cells [4–6]. BE exhibits free radical-scavenging activity and attenuates oxidative stress in cardiomyocytes [7,8]. However, some papers reported that the antioxidant activities could not be fully applied to explain the protective effects of flavonoids. Thus, more studies investigating the possible protective mechanisms are necessary. Our previous study indicated that BE treatment inhibited glioma C6 cells from oxidative stress-induced apoptosis in the presence of HO-1 protein induction [9]. Woo et al. indicated that BE protects rat cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism [10]. Although the protective effect of BE has been delineated, the relationship between HO-1 gene expression and the antioxidant/prooxidant activity in BE's protection against oxidative stress-induced apoptosis is still undefined.

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme to iron, carbon monoxide (CO), and biliverdin, and the biliverdin is then reduced by biliverdin reductase to produce bilirubin in mammals. Expression of the HO-1 gene is activated by a range of stimuli including prooxidants and antioxidants in various cell types [11,12]. Oxidative agents including heme, hyperoxia, and reactive oxygen species (ROS) have been shown to induce HO-1 gene expression through activation of mitogen-activated protein kinases (MAPKs) [13–16]. In addition, a range of dietary and naturally occurring antioxidants are considered to be beneficial because of their induction of HO-1 [17,18]. However, the regulatory mechanisms of these compounds have not been investigated. The roles of HO-1 induction have been investigated, and numerous studies have revealed the important functions of HO-1 as a cellular defense mechanism against oxidative insults. Lee et al. indicated that HO-1 plays a core role in the protective action of higenamine in ischemia–reperfusion-induced myocardial injury [19]. In an HO-1-overexpression experiment, cells expressed a cytoprotective effect against cisplatin-induced injury with reduced apoptosis [20]. In regard to four catalytic products of HO-1 including bilirubin, biliverdin, iron, and CO, both cytotoxic and cytoprotective effects of bilirubin and biliverdin have been reported [21–23], and CO has been shown to have vasodilatory, antiapoptotic, and anti-inflammatory properties [24–26]. Free iron has been shown to participate in deleterious oxidation reactions which stimulate ROS production, and HO-1 induction potentially contributes to a prooxidant state through the release of iron from heme. However, the roles of HO-1 and its four catalytic products in baicalein's protection against ROS-induced cytotoxic effects have not yet been elucidated.

Macrophages are vital for the recognition and elimination of microbial pathogens, and the survival of macrophages may directly contribute to the host defense system. Several previous studies showed that the virulence of some bacteria is due to their ability to trigger the death of activated macrophages via stimulating ROS production [27–29]. Therefore, investigating the protective mechanism in accordance with developing agents with ability to protect macrophages from ROS insults are important issues. In the present study, we assessed the role of the HO-1 protein's protective effect of baicalein against hydrogen peroxide (H_2O_2)-induced cell death in macrophages. Our results indicated that HO-1 induction via ROS-dependent ERK activation indeed plays an important role in the antiapoptotic effect of baicalein. The contribution of prooxidant rather than antioxidant effects to the cytoprotective activity of baicalein is identified.

2. Materials and methods

2.1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin), and 10% heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, MD) and maintained in a 37 °C humidified incubator containing 5% CO_2.

2.2. Agents

The structurally related flavonoids including baicalein, baicalin, quercetin, rutin, and quercitrin were obtained from Sigma Chemical (St. Louis, MO). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), H_2O_2, tin protoporphyrin (SnPP), N-acetyl cysteine (NAC), actinomycin D, cycloheximide, 2′,7′-dichlorodihydrofluorescein-diacetate (DCHF-DA), propidium iodine (PI), and 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) were purchased from Sigma. The Giemsa solution was purchased from Merck (Darmstadt, Germany). The anti-HO-1, anti-α-tubulin, anti-pERK, anti-pP38, anti-pJNK, and anti-PARP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, SB203580, and SP600125 were obtained from Calbiochem (La Jolla, CA).

2.3. Western blotting

Total cellular extracts were prepared according to our previous paper [30], separated on 8%~12% SDS-polyacrylamide minigels, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were incubated with 1% bovine serum albumin and then incubated with specific antibodies overnight at 4 °C. Expression of protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were treated with either BE for 6 h and then washed with ice-cold phosphate-buffered saline (PBS). Total RNA was isolated using a total RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the total RNA concentration was detected using a spectrophotometer. Total RNA (2 μg) was converted to cDNA with oligo d(T). PCR was performed on cDNA using the following respective sense and antisense primers for HO-1: CTGTGTAACC-TCTGCTGTTCC and CCACACTACCTGAGTCTACC, amplifying a 667-bp product; and for GAPDH: TGAAGGTCGGTGTGAACGGATTTGGC and CAT-GTAGGCCATGAGGTCCACCAC (983 bp). The PCR of the cDNA was performed in a final volume of 50 μl containing PCR primers, oligo (d)T, total RNA, and DEPC H_2O by RT-PCR beads (Amersham Pharmacia). The amplification sequence protocol was 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining [31].

2.5. Determination of ROS production

The production of reactive oxygen species (ROS) was monitored by flow cytometry using DCHF-DA. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide or low-molecular-weight hydroperoxides produced by cells oxidize DCHF to the highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. In the present study, cells were

treated with BE or BI for 2 h, with or without NAC (10 mM) pretreatment for 1 h, respectively. Then the compound-treated cells were washed twice with PBS to remove the extracellular compounds, and DCHF-DA (100 μM) green fluorescence was added, excited using an argon laser, and detected using a 525-nm (FL1-H) band-pass filter by a flow cytometric analysis [32].

2.6. Cell viability assay

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 12 h. Cells were washed with PBS three times, and MTT (50 mg/mL) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an enzyme-linked immunosorbent assay (ELISA) analyzer (Dynatech MR-7000; Dynex Technology, Chantilly, VA).

2.7. LDH release assay

The percentage of LDH release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in cells treated with 2% Triton X-100. The activity was monitored as the oxidation of NADH at 530 nm by an LDH assay kit (Roche).

2.8. DNA gel electrophoresis

Cells under different treatments were collected, washed with PBS twice, and lysed in 80 μL of lysis buffer (50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/mL proteinase K) for 3 h at 56 °C and then treated with 0.5 mg/mL RNase A for another hour at 56 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting point agarose, and 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gel containing 0.1 mg/mL ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer. The gels were observed and photographed under UV light [32].

2.9. Hypodiploid cell analysis

Cells were treated with the indicated compounds for a further 12 h. Trypsinized cells were washed with ice-cold PBS and were placed in 70% ethanol at −20 °C for at least 1 h. After fixation, cells were washed twice,

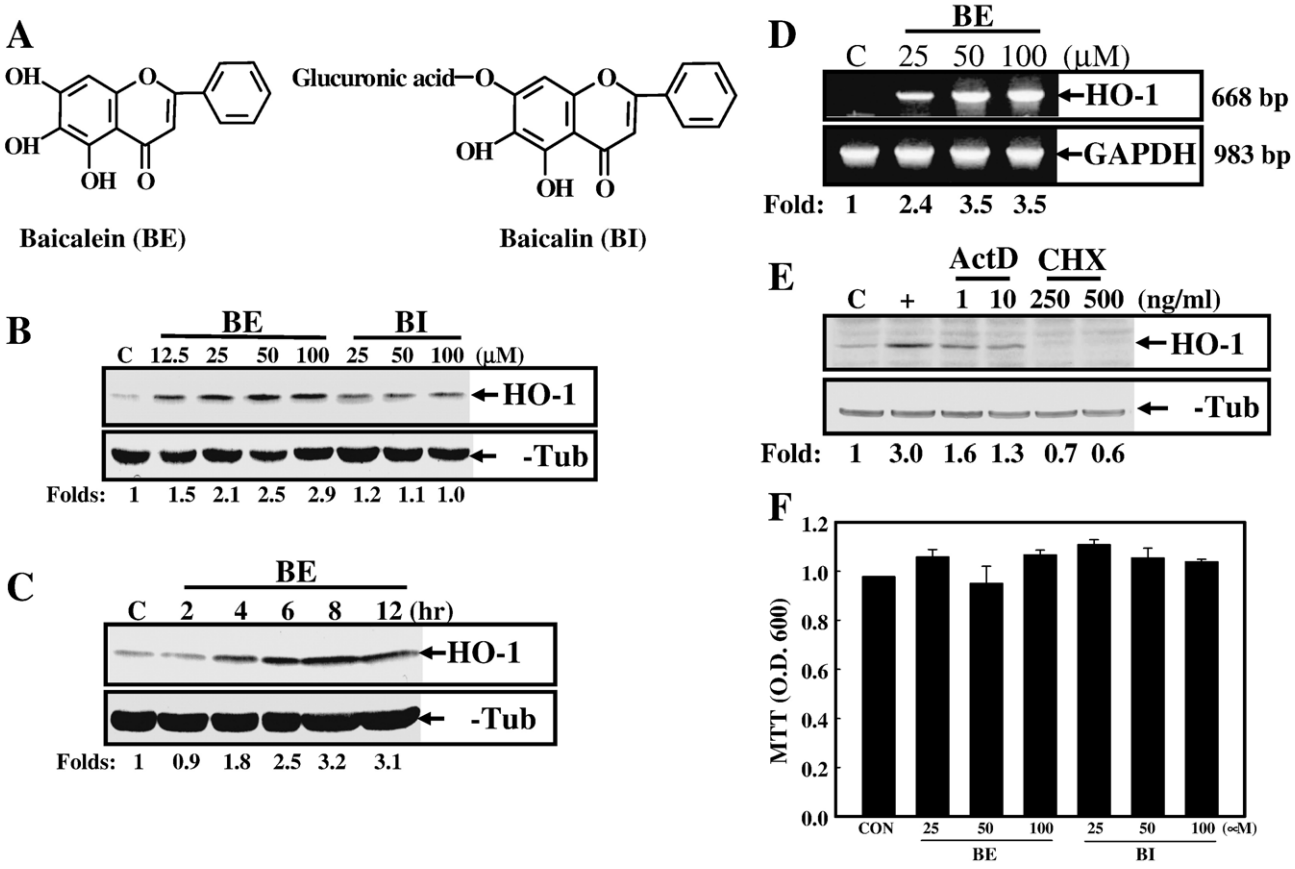


Fig. 1. Baicalein (BE), but not baicalin (BI), induction of HO-1 gene expression at both the protein and mRNA levels in RAW264.7 macrophages. (A) The chemical structures of BE and BI are described. BI contains an O-linked glucuronic acid at C7 of BE. (B) Dose-dependent induction of the HO-1 protein by BE. Cells were treated with different concentrations of BE (12.5, 25, 50, and 100 μM) or BI (25, 50, and 100 μM) for 12 h, and expression of the HO-1 protein was examined. (C) Time-dependent induction of the HO-1 protein by BE. RAW264.7 cells were treated with BE (50 μM) for 2, 4, 6, 8, and 12 h, and the expression of HO-1 protein was detected by Western blotting. (D) BE induction of HO-1 mRNA expression in macrophages. Cells were treated with BE (25, 50, and 100 μM) for 6 h, and the expression of HO-1 mRNA was examined by RT-PCR using specific primers. The expression of GAPDH mRNA was used as an internal control. (E) The addition of actinomycin D (ActD) and cycloheximide (CHX) inhibited BE-induced HO-1 protein expression. Cells were treated with BE (50 μM) in the presence or absence of ActD (1 and 10 ng/mL) or CHX (250 and 500 ng/mL) for 12 h, and expression of the HO-1 protein was examined. The expression of α-tubulin protein was used as an internal control. CON, control. (F) Neither BE nor BI affected the viability of macrophages. RAW264.7 cells were treated with different doses (25, 50, and 100 μM) of BE or BI for 12 h, and the viability of cells was examined by the MTT assay. CON, control. Quantification of intensity of each band was performed by densitometry analysis, and data were expressed as folds of control as described at the lower panel of figures.

incubated in 0.5 mL 0.5% Triton X-100/PBS at 37 °C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide (PI) for 10 min. Fluorescence emitted from the PI-DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickinson, San Jose, CA) [32].

2.10. Activities of caspase 3/CPP32 assay

After different treatments, cells were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 100 μg of protein were incubated with 100 μM enzyme-specific colorimetric substrates including Ac-DEVD-pNA for caspase 3/CPP32 at 37 °C for 1 h. Alternative activity of caspase 3 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm [32].

2.11. Measurement of the mitochondrial membrane potential

3,3′-Dihexyloxacarbocyanine iodide (DiOC6(3)) is a lipophilic cationic cyanine dye that occurs at the mitochondrial level and is widely used to determine the mitochondrial membrane potential. Cells were treated with BE or BI in the presence or absence of H_2O_2 for 6 h and then incubated with DiOC6(3) (40 nM) for 30 min at 37 °C. After treatment, cells were washed with ice-cold PBS, and trypsinized cells were washed with ice-cold PBS. Cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 500 μl of PBS. Fluorescence intensities of DiOC6(3) were analyzed on a flow cytometer (FACScan, Becton Dickinson) with excitation and emission settings of 484 and 500 nm, respectively.

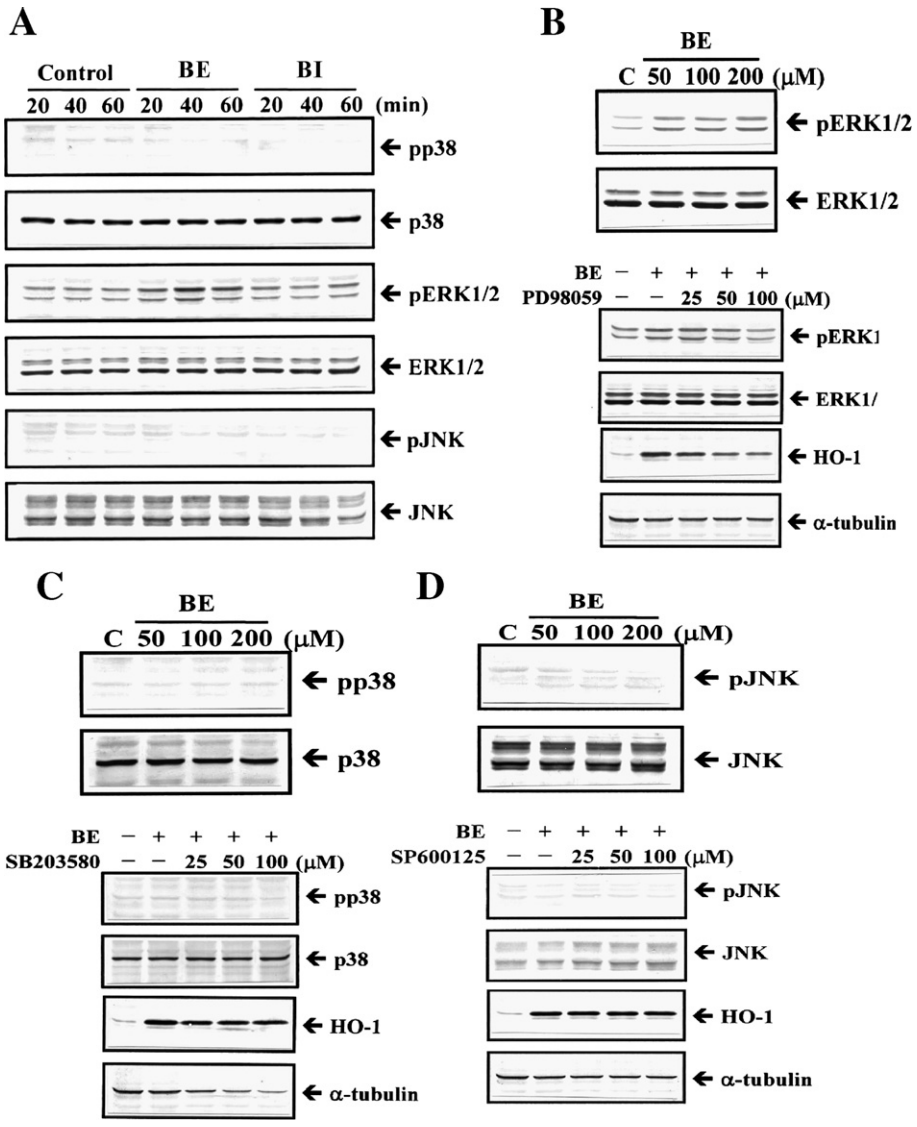


Fig. 2. Activation of ERKs is involved in baicalein (BE)-induced HO-1 protein expression. (A) A time-dependent induction of ERKs, but not p38 and JNK, protein phosphorylation in BE-treated macrophages. Cells were treated with BE or baicalin (BI) (50 μM) for 20, 40, and 60 min, and the expressions of the phosphorylated and total forms of p38, ERK, and JNK proteins were detected by Western blotting using specific antibodies. (B–D) (Upper panel) Dose-dependent induction of ERK (B), but not p38 (C) or JNK (D), protein phosphorylation was detected in BE-treated macrophages. Cells were treated with different concentrations (50, 100, and 200 μM) of BE for 40 min, and expressions of the phosphorylated and total forms of ERK, p38, and JNK proteins were detected by Western blotting. CON, control. (Lower panel) Effects of PD98059, SB203580, and SP600125 on ERK, p38, and JNK protein phosphorylation and HO-1 protein expression in the presence of BE (50 μM) treatment. Cells were treated with different doses (25, 50, and 100 μM) of PD98059, SB203580, and SP600125 for 30 min, followed by the addition of BE for an additional 40 min (for detecting the total and phosphorylated forms of the ERK, p38, and JNK proteins) or 12 h (for detecting HO-1 and α-tubulin protein expressions). Expression of the indicated protein was detected by Western blotting using specific antibodies. α-Tubulin was used as an internal control.

2.12. Cytochrome c release from mitochondria of RAW264.7 cells

Cells were treated with BE or BI in the presence of H_2O_2 for 12 h and harvested by centrifugation at 3000 rpm for 5 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended in five volumes of 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. Cells were homogenized and centrifuged at 1200 rpm for 10 min at 4 °C to separate them into supernatant and pellets. The supernatant was then centrifuged at 12,000 rpm for 15 min at 4 °C and the obtained supernatant was used for identification of cytosolic cytochrome *c* by immunoblotting. The pellets were lysed with 50 μl of lysis buffer consisting of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% SDS at 4 °C. The lysed solution was then centrifuged at 15,000 rpm for 30 min at 4 °C and used for the identification of mitochondrial cytochrome *c* by immunoblotting.

2.13. Establishment of HO-1 transfectants

pCMV-HO-1, a constitutive expression vector, carries full-length human HO-1 cDNA under control of the CMV promoter/enhancer sequence. We transfected pCMV-HO-1 or pCMV into RAW264.7 cells using the Transfast™ transfection reagent (Promega). After 48 h, cells were trypsinized and replated in DMEM with 10% FBS and 400 μg/mL G418. G418-resistant cells were selected and expanded. The level of HO-1 was analyzed by Western blotting [30].

2.14. Anti-sense HO-1 oligonucleotides

HO-1 sense and antisense oligonucleotides were directed against the flanking translation initiation codon and 6 base pairs on either side to the mouse HO-1 cDNA, and modified with phosphorothioate [33]. The sequence of the HO-1-specific antisense oligonucleotides was 5′-ACGCTCCATCACCGG-3′, and the sense oligonucleotides was 5′-CCGGTGATGGAGCGT-3′. Briefly, RAW264.7 macrophages were placed in serum-free medium and then transfected with the phosphorothioated HO-1-specific sense or antisense oligonucleotides (1 μg) for 48 h. After 48 h of incubation, cells were treated with BE for an additional 12 h, and the expression of HO-1 protein was analyzed by Western blotting.

2.15. Statistical analysis

Values are expressed as the mean±S.E. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A p value <0.05 or 0.01 was regarded as indicating a significant difference.

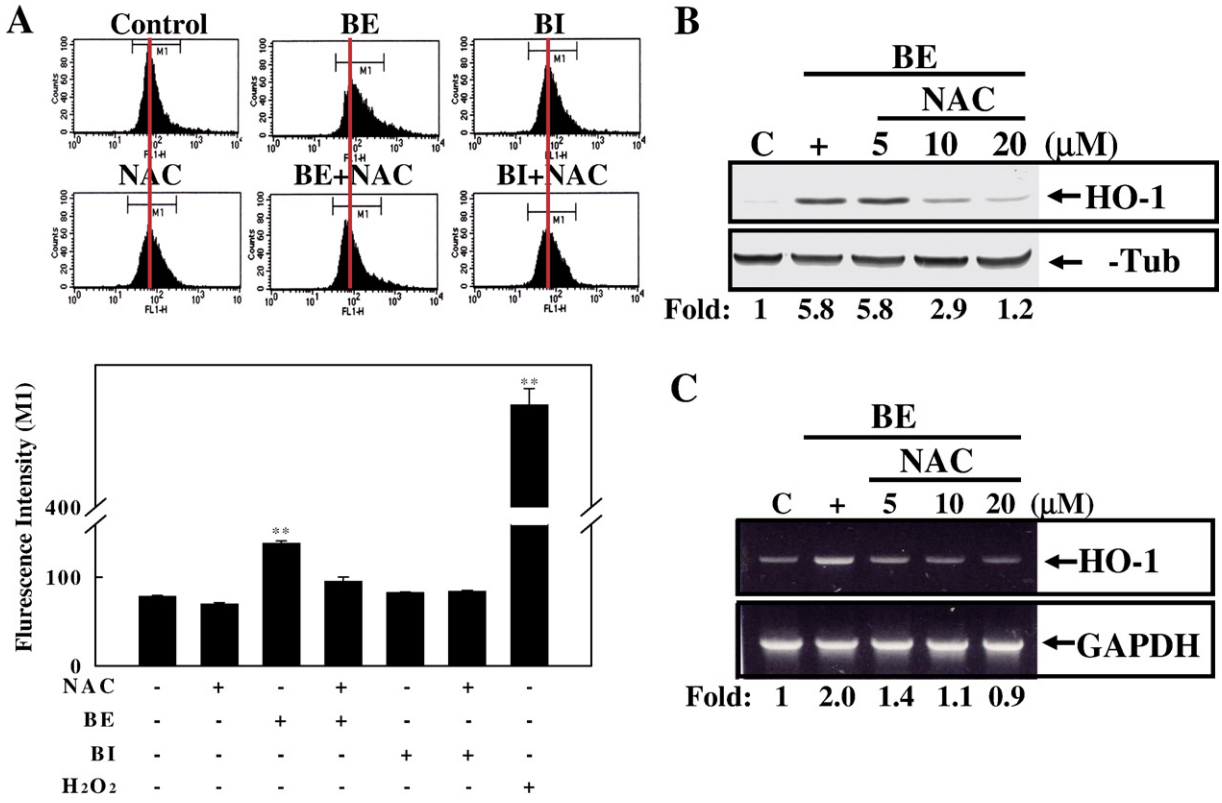
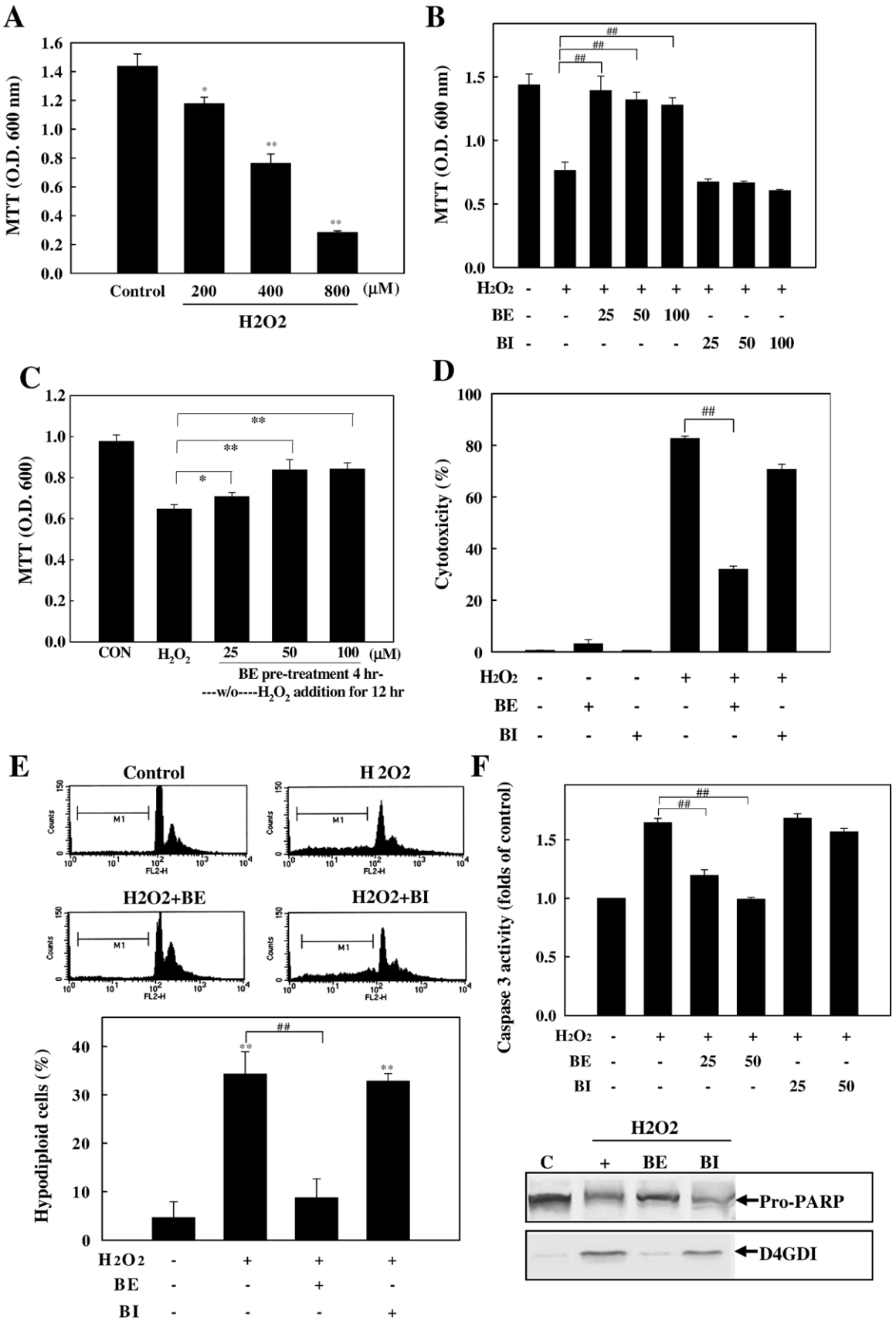


Fig. 3. Baicalein (BE) induced HO-1 gene expression via stimulating ROS production in macrophages. (A) BE stimulation of intracellular peroxide in macrophages using the DCHF-DA assay. Cells were treated with BE (50 μM) or baicalin (BI) (50 μM) for 1 h in the presence or absence of NAC (10 mM) pretreatment for 30 min. At the end of the reaction, DCHF-DA (100 μM) was added for an additional 1 h, and the DCF fluorescence intensity in cells was measured by a flow cytometric analysis. (Upper panel) A representative result of the flow cytometric analysis is provided. (Lower panel) Each value is presented as the mean±SE of the three independent experiments. $^{**}p < 0.01$ indicates a significant difference from the control, as analyzed by Student's *t*-test. The upper panel is a representative of the data of the flow cytometric analysis. (B) NAC prevention of BE-induced HO-1 expression. Cells were treated with BE for 12 h in the presence or absence of different concentrations (5, 10, and 20 mM) of NAC pretreatment for 1 h. Expression of the HO-1 protein was analyzed by Western blotting, and α-tubulin protein was used as an internal control. (C) NAC prevention of BE-induced HO-1 mRNA expression. Cells were treated with BE in the presence or absence of different concentrations (5, 10, and 20 mM) of NAC for 6 h, and the expression of HO-1 mRNA in each group was analyzed by RT-PCR. GAPDH was used as an internal control. Quantification of intensity of each band was performed by densitometry analysis, and data were expressed as folds of control as described at the lower panel of figures.


A
MTT (O.D. 600 nm)
Control
200
400
800
(μM)
H2O2
B
MTT (O.D. 600 nm)
H2O2
BE
BI
C
MTT (O.D. 600)
CON
H2O2
25
50
100
(μM)
BE pre-treatment 4 hr-
---w/o----H2O2 addition for 12 hr
D
Cytotoxicity (%)
H2O2
BE
BI
E
Control
H 2O2
H2O2+BE
H2O2+BI
Counts
FL2-H
M1
Hypodiploid cells (%)
H2O2
BE
BI
F
Caspase 3 activity (folds of control)
H2O2
BE
BI
H2O2
C
+
BE
BI
Pro-PARP
D4GDI

3. Results

3.1. Differential HO-1 induction by BE and its glycoside, BI, in RAW264.7 macrophages

The chemical structures of BE and BI are shown in Fig. 1A. BE and BI are structurally related flavonoids, with BI possessing a glucuronic acid at the C7 of BE. Results of Fig. 1B and C show that BE but not BI induced HO-1 protein expression in concentration- (Fig. 1B) and time-dependent (Fig. 1C) manners. The plateau of BE-induced HO-1 protein was observed at BE concentrations between 50 and 100 μM for 12 h incubation times, and at the times between 8 and 12 h after BE (50 μM) treatment. In order to determine if induction of HO-1 gene expression by BE is regulated at the transcriptional level, RT-PCR using specific primers for HO-1 and GAPDH was performed. Results in Fig. 1D show that BE induced HO-1 gene expression at the mRNA level in a dose-dependent manner. Actinomycin D (ActD) and cycloheximide (CHX) are inhibitors of de novo transcription and translation, respectively. Results in Fig. 1E show that HO-1 protein induction by BE was significantly blocked by the addition of ActD (1 and 10 ng/ml) and CHX (0.25 and 0.5 μg/ml). These data indicate that de novo protein synthesis is essential for BE's induction of HO-1 gene expression. Additionally, neither BE nor BI exhibited cytotoxic effects on RAW264.7 cells according to the MTT assay (Fig. 1F). This suggests that BE is an effective HO-1 inducer without cytotoxicity in RAW264.7 macrophages.

3.2. BE induction of HO-1 gene expression via activation of ERKs in macrophages

Activation of intracellular kinases such as MAPKs has been shown in the regulation of the expression of several genes. To examine if activation of MAPKs is involved in BE's induction of HO-1 protein expression, RAW264.7 cells were treated with BE or BI (50 μM) for 20, 40, and 60 min, and expressions of the three phosphorylated MAPK proteins including ERK, JNK, and p38 protein were examined by Western blotting using specific antibodies. Results in Fig. 2A show that BE but not BI time-dependently induced phosphorylated ERK, but not JNK or p38, protein expression. Similarly, BE dose-dependently induced phosphorylated ERK, but not JNK or p38, protein expression in RAW264.7 cells (Fig. 2B–D; upper panel). We further explored if activation of ERK is an essential event in BE's induction of HO-1 protein expression via a pharmacological study applying three well-known inhibitors of MAPKs including PD98059 (an ERK inhibitor), SB203580 (a p38 kinase inhibitor), and SP600125 (a JNK inhibitor). Data of Fig. 2B–D (lower panels) showed that the addition of PD98059 dose-dependently inhibited BE-induced phosphorylation of ERK proteins with a decrease in HO-1 protein expression. Neither SB203580 nor SP600125 showed an inhibitory effect on HO-1 protein expression induced by BE. These data suggest that HO-1 is induced by BE via activation of ERKs in macrophages.

3.3. BE-induced HO-1 gene expression via a ROS-dependent manner in RAW264.7 cells

In order to investigate if ROS production is involved in BE's induction of HO-1 gene expression, intracellular peroxide levels were examined by a DCHF-DA assay. A significant increase in intracellular peroxide levels was detected in BE-treated cells, and the addition of the chemical antioxidant, NAC, significantly reduced intracellular peroxide production induced by BE. In contrast to BE, no change in the intracellular peroxide level was found in BI-treated cells (Fig. 3A). A significant increase in intracellular peroxide level by H_2O_2 was used as a positive control. Additionally, HO-1 gene expression at both the protein and mRNA levels induced by BE was blocked by the addition of NAC to RAW264.7 cells (Fig. 3B, C). These data indicate that ROS induction is involved in BE-induced HO-1 gene expression in macrophages.

3.4. BE, but not BI, protection of RAW264.7 cells from H_2O_2-induced apoptosis

We further analyzed the protective effects of BE and BI on H_2O_2-induced cytotoxicity in RAW264.7 cells. In the presence of H_2O_2, a dose-dependent decrease in the viability of cells was observed by the MTT assay in RAW264.7 cells, with an IC_{50} value of around 400 μM (Fig. 4A). Interestingly, in the

Fig. 4. The addition of baicalein (BE), but not baicalin (BI), significantly attenuated H_2O_2-induced cell death in macrophages. (A) Dose-dependent reduction of the viability [of macrophages?] by H_2O_2 using the MTT assay. Cells were treated with different doses of H_2O_2 (200, 400, and 800 μM) for 12 h, and the viability of macrophages was detected by the MTT assay as described in Materials and methods. (B) BE inhibition of H_2O_2-induced cell death using the MTT assay. Cells were treated with different concentrations (25, 50, and 100 μM) of BE or BI for 30 min followed by the addition of H_2O_2 (400 μM) for a further 12 h, and the viability of cells in each group was detected by the MTT assay. (C) Long-term treatment of BE inhibited H_2O_2-induced cell death. Cells were pretreated with BE or BI (25 and 50 μM) for 4 h, and washed twice with PBS to remove BE in the medium, followed by H_2O_2 (400 μM) treatment for a further 12 h. The viability was detected by the MTT assay. (D) BE inhibition of H_2O_2-induced LDH release in the culture medium. Cells were treated with BE or BI (50 μM) for 30 min followed by the addition of H_2O_2 for a further 12 h. The amount of LDH in the medium was detected as described in Materials and methods. The amount of total LDH was detected by adding 1% Triton X-100 to the macrophages. The percentage of cytotoxicity is expressed by the equation: [(Tested group − Control group)/(Triton X-100-group − Control group)] × 100%. (E) BE but not BI inhibited H_2O_2-induced hypodiploid cells (sub-G1) using a flow cytometric analysis. Macrophages were treated with BE or BI (50 μM) followed by H_2O_2 (400 μM) treatment. The ratio of hypodiploid cells was detected by PI-staining via a flow cytometric analysis. (Upper) A representative result of the flow cytometric analysis; (lower) the quantitative data derived from three independent experiments. (F) BE, but not BI, inhibited caspase 3 enzyme activity and the cleavage of PARP and D4-GDI proteins induced by H_2O_2. As described previously, cells were treated with BE or BI (25 and 50 μM) for 30 min followed by H_2O_2 treatment for 12 h, and the activity of caspase 3 was examined using a caspase 3-specific colorimetric substrate, Ac-DEVD-pNA (upper panel). (Lower panel) Expressions of the pro-form of the PARP protein and cleaved form of the D4-GDI protein were examined by Western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$ indicate a significant difference from the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ indicate a significant difference between designated groups, as analyzed by Student's *t*-test.

presence of BE, but not BI, with H_2O_2, BE significantly attenuated the H_2O_2-induced cytotoxicity according to the MTT assay (Fig. 4B). As illustrated in Fig. 1B, the HO-1 protein induced by BE was initially detected at 4 h post-treatment. Therefore, in the condition of cells pretreated with BE for 4 h followed by the addition of H_2O_2, the H_2O_2-induced cytotoxic effects were also significantly reduced in the presence of BE treatment according to the MTT assay (Fig. 4C). BE protection of cells from H_2O_2-induced cytotoxic effects was also identified by the LDH (lactate dehydrogenase) release assay (Fig. 4D). In addition, reduction of H_2O_2-induced hypodiploid cells by BE, but not BI, was detected by a flow cytometric analysis (Fig. 4E). Elevation of caspase 3 enzyme activity in accordance with a reduction in pro-PARP protein and an increase in cleaved D4-GDI protein expression by H_2O_2 was examined in macrophages, and those events were blocked by BE, but not BI, treatment (Fig. 4F).

3.5. Attenuation of the protective effect of BE against H_2O_2-induced cytotoxicity by the HO-1 enzyme inhibitor, SnPP, or ERK inhibitor, PD98059

We further determined if the HO-1 protein is involved in the protective mechanism of BE against H_2O_2-induced cytotoxicity. SnPP is a well-known HO enzyme inhibitor, and inhibits the conversion of hemin to bilirubin. As illustrated in Fig. 5A, SnPP alone showed no effect on H_2O_2-induced cytotoxicity, whereas the protective effect of BE against H_2O_2-induced cytotoxicity was attenuated by the addition of SnPP. PD98059, shown to suppress BE-induced HO-1 gene expression in Fig. 2,

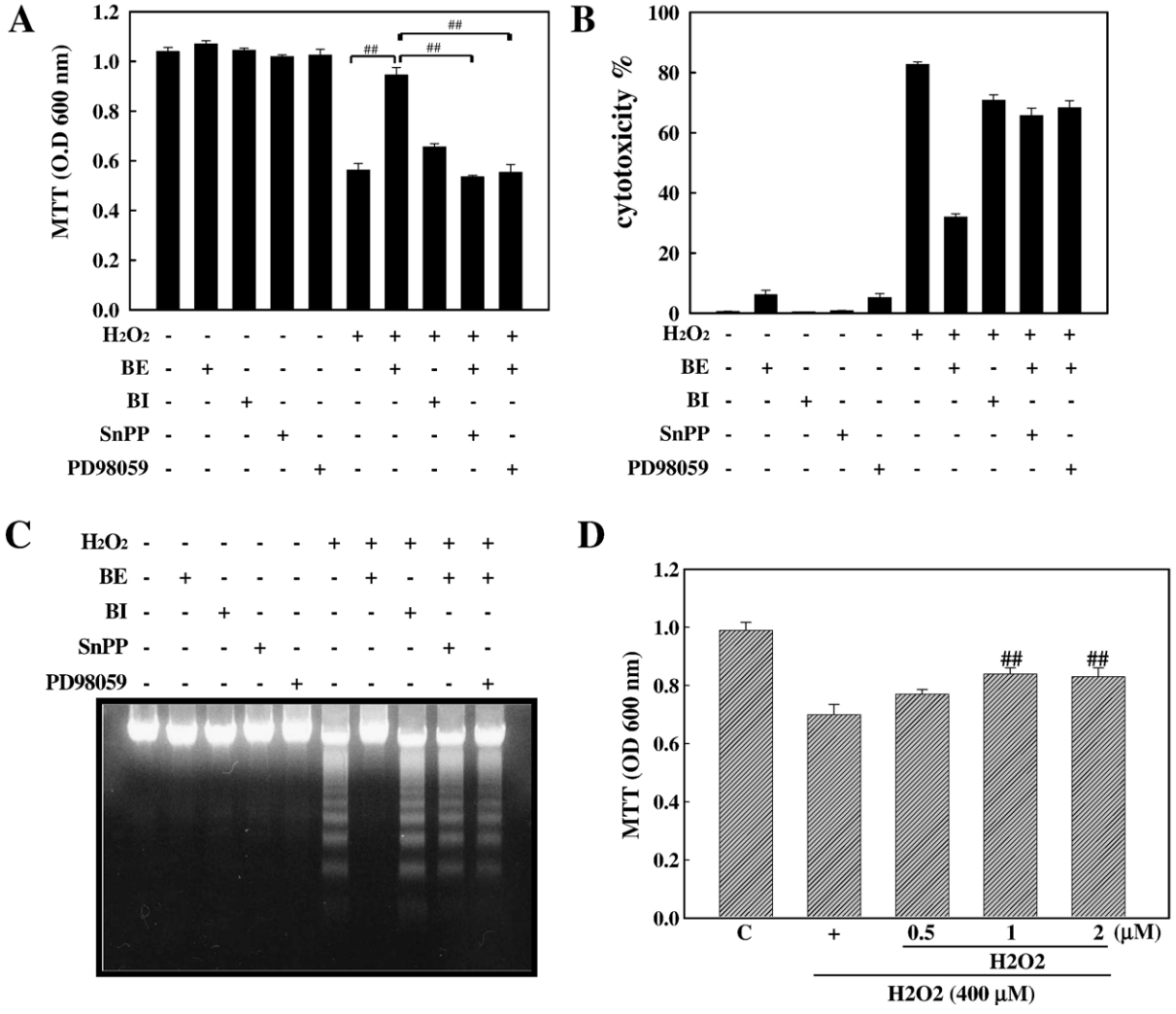


Fig. 5. SnPP and PD98059 attenuated the inhibitory effect of BE against H_2O_2-induced apoptosis. (A) RAW264.7 cells were treated with baicalein (BE) or baicalin (BI) (50 μM) with or without pretreatment with SnPP (20 μM) and PD98059 (20 μM) followed by H_2O_2 incubation for 12 h. The viability of cells under different treatments was analyzed by MTT (A), and LDH release assays (B). $^{\#\#}p < 0.01$ indicates a significant difference between the designated groups. (C) As described in panel (A), the integrity of DNA with the appearance of DNA ladders in each group was analyzed via agarose electrophoresis. (D) Pre-challenge of cells with lower concentrations (0.5, 1, and 2 μM) of H_2O_2 may reverse the cytotoxicity elicited by H_2O_2 (400 μM). Cells were pretreated with lower concentrations (0.5, 1, and 2 μM) for 4 h, and washed twice with PBS to remove H_2O_2 in the medium, followed by H_2O_2 (400 μM) treatment for a further 12 h. The viability of cells was detected by the MTT assay. Data are expressed as the mean ± SE. $^{\#\#}p < 0.01$ indicates a significant difference from H_2O_2-treated group.

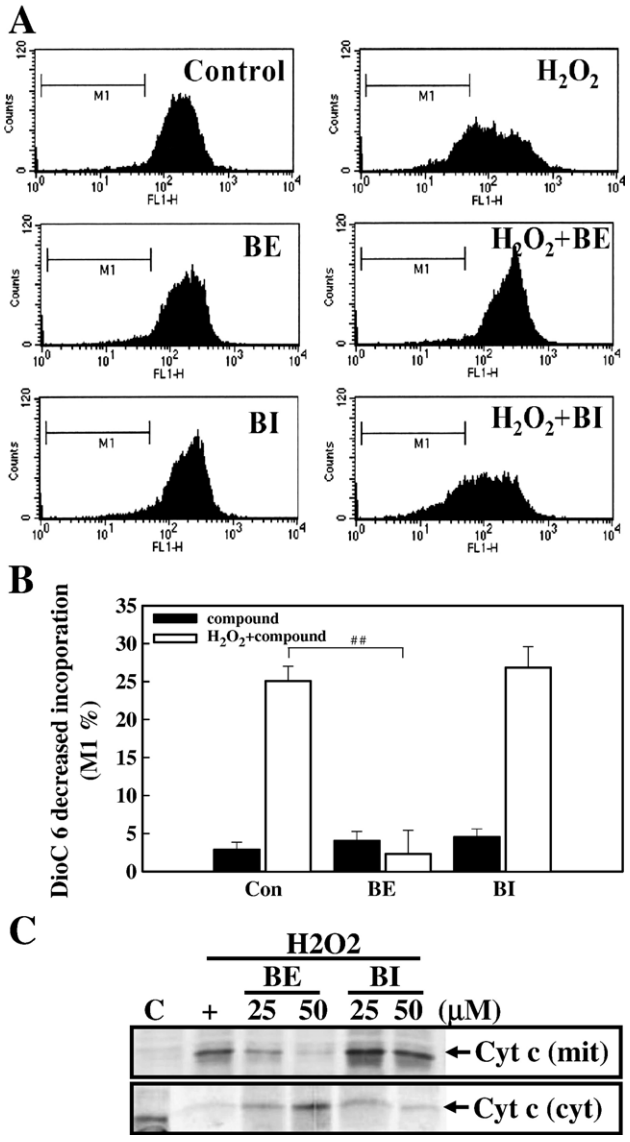


Fig. 6. Baicalein (BE) inhibited the H_2O_2-induced loss in mitochondrial membrane potential. (A) Cells were pretreated with BE or baicalin (BI) (50 μM) for 30 min followed by incubation with H_2O_2 (400 μM) for 6 h, and incubation of cells with DiOC6 (100 μM) for an additional 30 min. The fluorescence intensity of cells was measured by a flow cytometric analysis. A representative example of the flow cytometric analysis is shown. (B) Data derived from three independent experiments were analyzed, and are expressed as the mean ± SE. $^{\#\#}p < 0.01$ indicates a significant difference between the indicated groups, as analyzed by Student's *t*-test. (C) BE inhibited the H_2O_2-induced release of the cytochrome *c* protein from mitochondria to the cytosol. Cells were treated with different doses (25 and 50 μM) of BE or BI for 30 min followed by the addition of H_2O_2 (400 μM) for 6 h. The expression of cytochrome *c* in both cytosolic (Cyt) and mitochondrial fractions (Mit) was detected by Western blotting.

significantly inhibited the protective effect of BE against H_2O_2-induced cytotoxicity according to the MTT assay. Attenuation of BE-induced protection by SnPP and PD98059 was also identified by LDH release assays (Fig. 5B). Furthermore, analysis of DNA integrity indicated the occurrence of DNA ladders in H_2O_2-treated cells, which was inhibited by the addition of BE but not BI. BE's action against H_2O_2-induced DNA ladders was significantly inhibited by SnPP or PD98059 incubation. Neither BE, BI, SnPP, nor PD98059 exhibited an ability to induce DNA ladders in the absence of H_2O_2 (Fig. 5C). In order to confirm ROS-dependent protection in macrophages, cells were treated with lower concentrations (0.5, 1, and 2 μM) of H_2O_2 for 4 h, followed by H_2O_2 (400 μM) stimulation. Data in Fig. 5D showed that incubation of RAW264.7 cells with lower concentrations of H_2O_2 was able to prevent cells from the following H_2O_2 (400 μM)-induced cytotoxicity via MTT assay. These data imply that BE reduction of cytotoxicity induced by H_2O_2 is mediated through blocking of the induction of apoptosis, and induction of HO-1 gene expression may be involved.

3.6. BE inhibits H_2O_2-induced reduction of the mitochondrial membrane potential in RAW264.7 cells

Alterations in the mitochondrial membrane potential under different treatments were evaluated by flow cytometric analysis using DiOC6 as the fluorescence indicator. As illustrated in Fig. 6A and B, BE and BI showed no effect on the mitochondrial membrane potential in RAW264.7 cells in the absence of H_2O_2. A

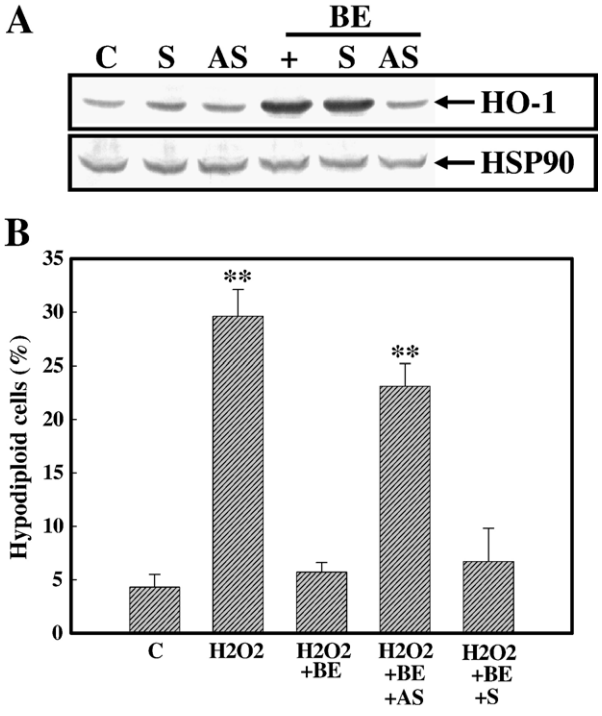


Fig. 7. Suppression of HO-1 gene expression by the HO-1 antisense oligonucleotide significantly inhibited the protective effect of baicalein (BE) against H_2O_2-induced apoptosis. (A) Transfection of the HO-1 antisense, but not sense, oligonucleotide reduced the expression of HO-1 protein induced by BE in macrophages. Cells were transfected with 1 μg of HO-1 antisense or sense oligonucleotide, followed by the addition of BE (50 μM) for an additional 12 h. The expression of HO-1 protein was examined by Western blotting. C, control group; S, HO-1 sense oligonucleotide; A, HO-1 antisense oligonucleotide. (B) The HO-1 antisense oligonucleotide attenuated the antiapoptotic effect of BE against H_2O_2. Cells were transfected with antisense or sense oligonucleotide, followed by BE (50 μM) treatment for 30 min with or without an additional H_2O_2 treatment for 12 h. The ratio of hypodiploid cells in each group was examined by a flow cytometric analysis as described in Fig. 4.

significant reduction in the mitochondrial membrane potential was detected in H_2O_2-treated cells, and this was prevented by the application of BE but not BI. Cytochrome c is a mitochondrial protein, and release of cytochrome *c* to the cytosol has been shown to be a marker of mitochondrial dysfunction. As shown in Fig. 6C, the release of cytochrome *c* from mitochondria to the cytosol was detected in H_2O_2-treated macrophages, and this was blocked by the addition of BE but not BI. This suggests that BE possesses the ability to suppress loss of the mitochondria membrane potential induced by H_2O_2.

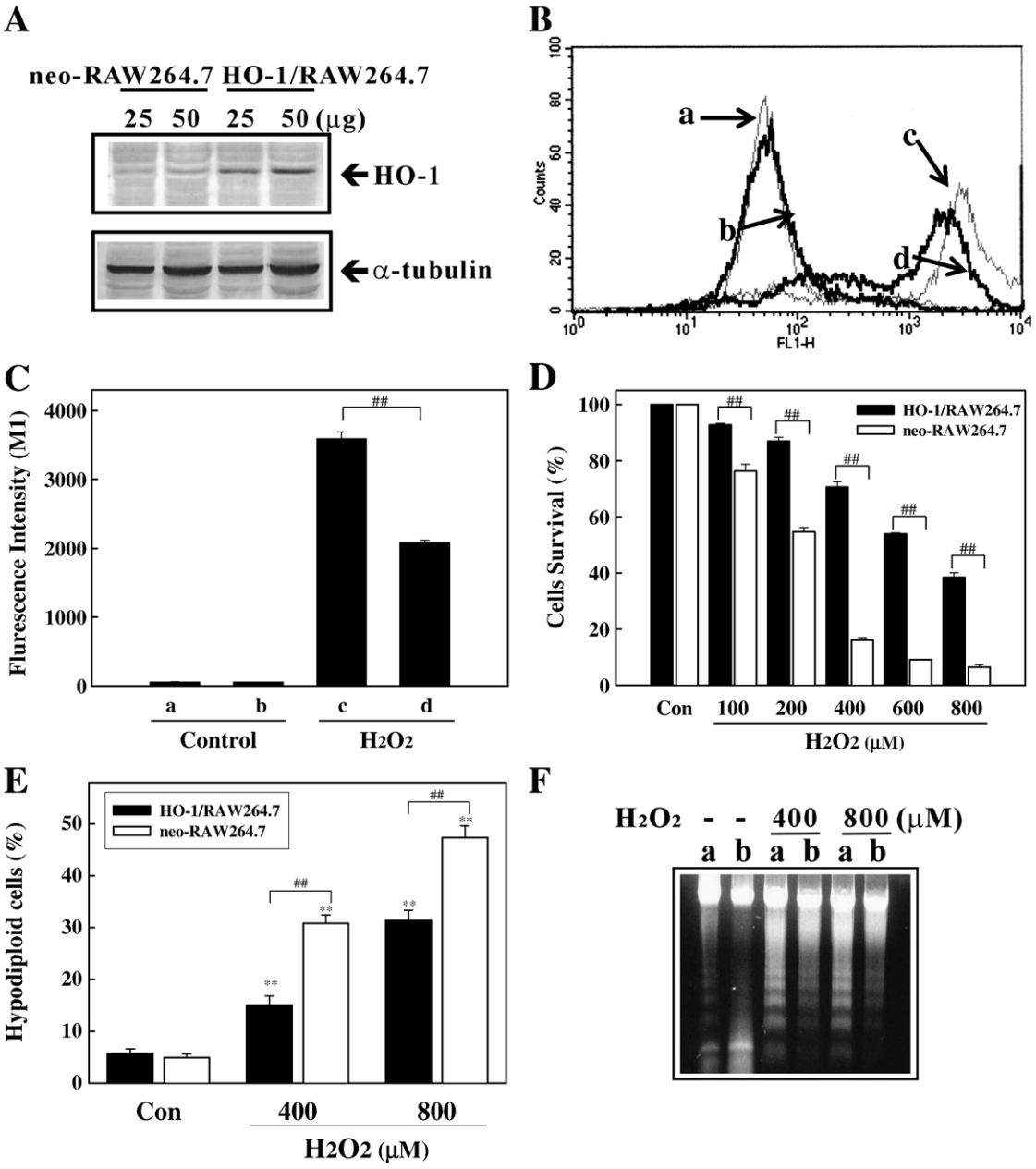


Fig. 8. Overexpression of the HO-1 protein via stable transfection of the HO-1 expression vector attenuated H_2O_2-induced apoptosis through reducing intracellular peroxide production by macrophages. (A) An increase in the intracellular HO-1 protein via transfection of HO-1 expression vector. Cells were transfected with a Neo-control vector or an HO-1-expressing vector as described in Materials and methods, and expression of the HO-1 protein was detected by Western blotting. (B) Overexpression of the HO-1 protein decreased the intracellular peroxide level induced by H_2O_2. Both neo-RAW264.7 (gray curve) and HO-1/RAW264.7 (black curve) cells were incubated in conditions with or without H_2O_2, and the level of intracellular peroxide was examined by the DCHF-DA assay via a flow cytometric analysis. a, Neo-RAW264.7 cells; b, HO-1/RAW264.7 cells; c, H_2O_2-treated Neo-RAW264.7 cells; d, H_2O_2-treated HO-1/RAW264.7 cells. A representative example of the flow cytometric analysis is presented. (C) Data in B were obtained from three independent experiments, and are presented as the mean ± SE. $^{\#\#}p < 0.01$ indicates a significant difference from the compared group, as analyzed by Student's *t*-test. (D) Neo-RAW 264.7 cells were more sensitive to H_2O_2 challenge than were HO-1/RAW264.7 cells. Both cells were treated with different doses of H_2O_2 (100, 200, 400, 600, and 800 μM) for 12 h, and the viability of cells was examined by the MTT assay. (E) As described in (D), the ratio of hypodiploid cells in both cells in the presence of H_2O_2 (400 and 800 μM) treatment was calculated by a flow cytometric analysis. (F) As described in (E), the integrity of DNA in each group was analyzed by agarose electrophoresis.

3.7. HO-1 protein indeed participates in BE's protection against H_2O_2-induced cytotoxicity via reducing ROS production

In order to provide direct evidence to demonstrate if the HO-1 protein participates in BE's prevention of H_2O_2-induced cytotoxicity in macrophages, an antisense HO-1 oligonucleotide transfection experiment and establishment of a stable HO-1-overexpressed macrophage were performed in this study. As illustrated in Fig. 7A and B, transfection of an antisense, but not sense, HO-1 oligonucleotides in RAW264.7 cells significantly reduced HO-1 protein expression induced by BE in accordance with inhibiting the preventive effect of BE against H_2O_2-induced hypodiploid cells by flow cytometric analysis. In the HO-1 overexpression experiments, both HO-1-overexpressing (HO-1/RAW264.7) and neo-controlled (neo/RAW264.7) RAW264.7 cells were established via a G418 selection method as described in our previous study [29]. As illustrated in Fig. 8A, expression of HO-1 protein in HO-1/RAW264.7 cells was much higher than that in Neo/RAW264.7 cells. We further analyzed the level of intracellular peroxide in the presence of H_2O_2 stimulation in both cells by a flow cytometric analysis via DCHF-DA staining. Data of Fig. 8B and C show that H_2O_2-induced peroxide levels in HO-1/RAW264.7 cells (2081.20±32.97) were lower than those in Neo/RAW264.7 cells (3591.68±97.56). In the same part of the experiment, the percentages of H_2O_2-induced cytotoxicity and hypodiploid cells were reduced in HO-1/RAW264.7 cells, in comparison with those in Neo/RAW264.7 cells (Fig. 8D, E). Electrophoretic analysis of DNA integrity also showed that the intensity of DNA ladders was reduced in HO-1/RAW264.7 cells, in comparison with that in neo/RAW264.7 cells (Fig. 8F).

3.8. Carbon monoxide (CO) possesses the ability to inhibit H_2O_2-induced cytotoxicity

HO-1 induction may catalyze the cleavage of the α-meso carbon bridge of heme, yielding three products including CO, biliverdin, and free iron. In order to evaluate if these products participate in the preventive effect of HO-1 against H_2O_2-

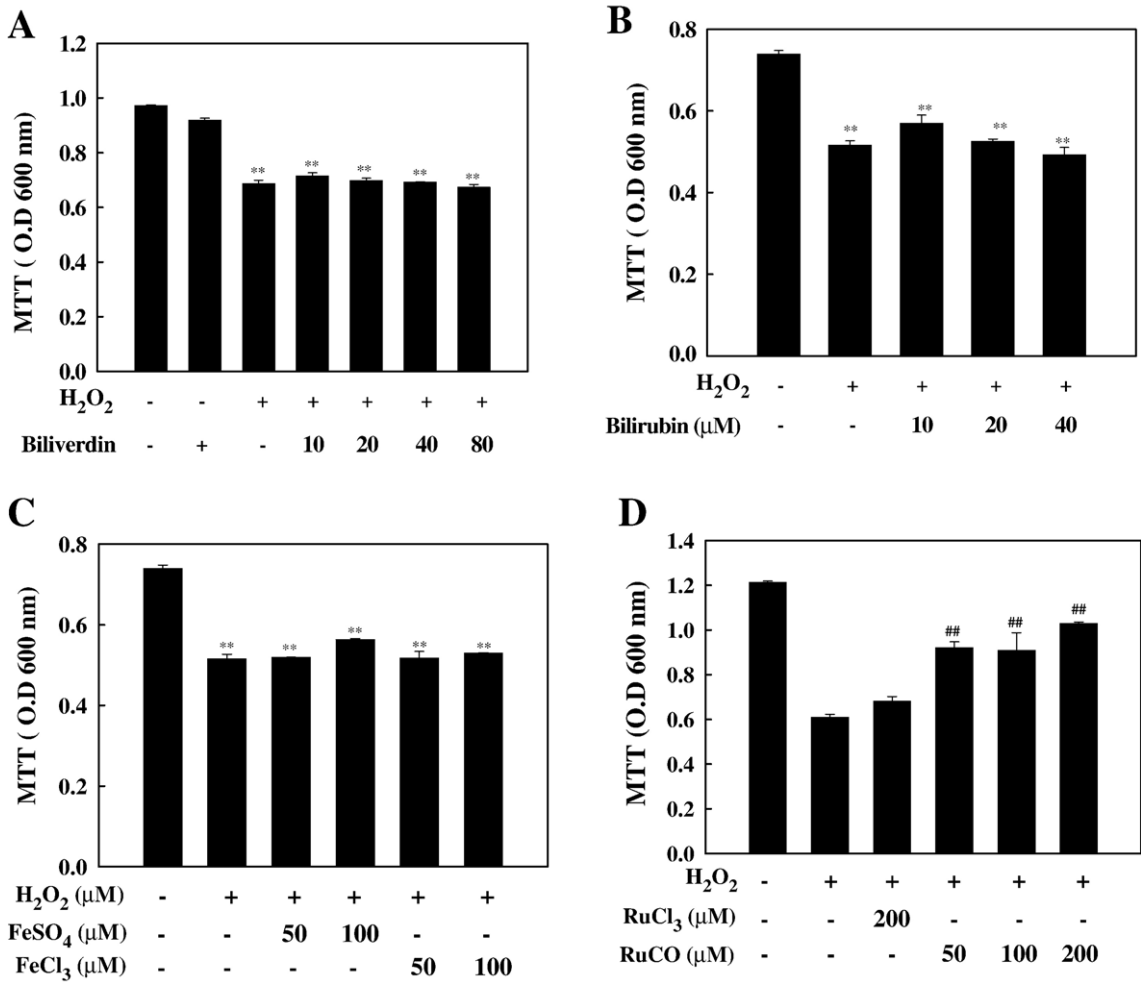


Fig. 9. Addition of a CO donor, RuCO, but not RuCl3, bilirubin, biliverdin, $FeCl_3$, or $FeSO_4$, significantly reduced H_2O_2-induced cell death in macrophages. Production of CO, bilirubin, biliverdin, and ferric ion has been shown in HO-1-catalyzed heme metabolism. Cells were treated with biliverdin (A: 10, 20, 40, and 80 μM), bilirubin (B: 10, 20, and 40 μM), $FeCl_3$ (C: 50 and 100 μM), $FeSO_4$ (C: 50 and 100 μM), RuCO (D: 50, 100, and 200 μM), and $RuCl_3$ (200 μM) for 30 min, followed by incubation with H_2O_2 (400 μM) for an additional 12 h. The viability of cells in the different groups was evaluated by the MTT assay. $^{\#\#}p < 0.01$ indicates a significant difference from the H_2O_2-treated group, as analyzed by Student's *t*-test.

induced insults, RAW264.7 macrophages were treated with different doses of biliverdin, bilirubin, $FeCl_3$, $FeSO_4$, and the CO donor, RuCO, and its reference compound, $RuCl_3$, followed by H_2O_2 treatment, and the viability of cells was detected with the MTT assay. As illustrated in Fig. 9A–C, application of biliverdin, bilirubin, $FeCl_3$, and $FeSO_4$ did not affect the cytotoxicity induced by H_2O_2. The addition of RuCO but not $RuCl_3$ significantly and dose-dependently inhibited the H_2O_2-induced cytotoxicity in RAW264.7 macrophages (Fig. 9D).

4. Discussion

Both BE and BI are potent antioxidants through their formation of stable semiquinone radicals. Miura et al. investigated ROS generation by flavonoids, and indicated that BE possesses the ability to generate H_2O_2 after 4 h of incubation [34]. These data suggest that flavonoids are able to auto-oxidize in aqueous conditions with the production of H_2O_2, and a decrease in their antioxidant or stimulation of their prooxidant effects can be observed. In the present macrophage culture system, no cytotoxic effects of BE or BI were observed at 100 μM, and the addition of BE significantly protected macrophages from H_2O_2-induced apoptosis in accordance with stimulation of HO-1 gene expression and ROS production. Woo et al. [10] also reported that BE protects cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism [10], however they did not elucidate the mechanism. Our data provide an explanation of how a slight but significant increase in ROS production by BE may act as a signal molecule to activate intracellular kinase cascades which in term induce cytoprotective gene expression (such as the HO-1 gene), that may contribute to the antiapoptotic effect of BE. It is an important finding which leads us to speculate on the beneficial side of the prooxidant effect related to cytoprotection.

The structure–activity relationship of flavonoids is still undetermined. Several studies have indicated that flavonoids with a greater number of hydroxyl substitutions show more-significant antioxidant and prooxidant activities, and hydroxylation at C3' and C4' of the B ring and a 2,3-double bond in conjugation with a 4-oxo group in the C ring are crucial for the antioxidant activity of flavonoids [35]. Additionally, the catechol moiety in the B ring of flavonoids has been shown to bind with ferric and copper ions to reduce ROS production [36]. Glycosylation commonly occurs in the metabolism of flavonoids to increase their hydrophilicity, and several previous studies indicated that glycosides significantly affect the biological activities of flavonoids [37,38]. Quercetin expressed more-potent apoptosis-inducing activity than its glycosides, rutin and quercitrin [39], and hesperitine, but not its glycoside, hesperidine, significantly inhibited LPS-induced NO production and iNOS gene expression in macrophages [40]. In the present study, BE, but not its glycoside, BI, exhibited the ability to protect macrophages from H_2O_2-induced apoptosis through induction of the HO-1 protein. These data support the notion that the sugar moiety plays a negative role in flavonoids' prevention of apoptosis.

ROS have been shown to be involved in maintaining human physiological functions, however large amounts of ROS are detrimental and have been shown to participate in the etiology of several human diseases such as cancer, inflammation, and diabetes. Therefore, the further development of agents with the ability to block damage induced by detrimental amounts of ROS has recently been receiving greater attention. Both ROS-scavenging (antioxidant) and ROS-producing (prooxidant) activities of flavonoids have been reported [41]. When acting as prooxidants, flavonoids can stimulate apoptotic events such as proteolytic cleavage of PARP, induction of DNA ladders, and loss of the mitochondrial membrane potential, accompanied by accumulation of ROS and depletion of intracellular GSH levels [42,43]. In the present culture system, we observed that BE incubation enhanced intracellular peroxide levels in the DCHF-DA assay in accordance with stimulation of HO-1 gene expression through activation of ERKs, and NAC treatment inhibited BE-induced HO-1 gene expression and ERK protein phosphorylation by reducing intracellular peroxide production. Interestingly, pre-incubation with BE for both 30 min and 8 h significantly reduced subsequent H_2O_2-induced cell death. These data imply that BE's prevention of oxidative stress-induced apoptosis may be through its direct antioxidant activity or an indirect effect via ROS-dependent stimulation of an intracellular signaling cascade which activates the HO-1 cellular defense gene.

HO-1 overexpression dramatically attenuates pathological activities including inflammation, vascular proliferation, and decreased chronic transplant rejection [44,45]. Our previous study demonstrated that both NO and PGE_2 are potent inducers of the HO-1 gene [46], and that overexpression of the HO-1 protein inhibits lipopolysaccharide-induced iNOS expression and NO production [30,40]. Zhang et al. indicated that the exogenous HO-1 gene within vascular smooth muscle cells (VSMCs) protects them from free radical attack and inhibits cell proliferation [47]. Tobiasch et al. also suggested that tumor necrosis factor (TNF)-α decreases the percentage of apoptotic cells in pancreatic beta cells [48]. Lee et al. demonstrated that the HO-1 protein is essential for the anti-inflammatory effects of IL-10 and 15-deoxy-delta 12,14-prostaglandin J2 [49,50]. In the present study, the addition of the chemical HO-1 inhibitor, SnPP, or an HO-1 antisense oligonucleotide reduced the protective effect of BE against H_2O_2-induced macrophage cell death, and exogenous overexpression of the HO-1 protein prevented macrophages from H_2O_2-induced apoptosis through a reduction in intracellular ROS levels. This suggests that HO-1 plays an important role as a target in macrophages against ROS-mediated damage, and flavonoids with the ability to induce HO-1 gene expression may act as protectors against oxidative stress.

Many actions of biliverdin, iron, and CO have been reported. CO has vasodilatory effects, and inhalation of CO has been shown to protect tissues against hyperoxia [51]. The iron released by HO-1-mediated heme degradation can catalyze free radical reactions which stimulate ROS production. Additionally, both cytotoxic and cytoprotective properties of bilirubin have been identified. Seubert et al. reported that bilirubin induces apoptosis in Hepa1c1c7 hepatoma cells [52]. In other papers, the protective effects of bilirubin against oxidative stress have been shown in several types of cells including VSMCs and endothelial cells [53–55]. Tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$) has

been shown to rapidly elicit CO formation when added directly to a solution. Motterlini et al. indicated that the amount of MbCO formed was dependent on the concentration of $[Ru(CO)_3Cl_2]_2$ used, and each mole of $[Ru(CO)_3Cl_2]_2$ may produce approximately 0.7 moles of CO by spectrophotometric assay [56]. $[Ru(CO)_3Cl_2]_2$ caused sustained vasodilation in precontracted rat aortic rings and attenuated coronary vasoconstriction in hearts ex vivo, and those vascular effects were mimicked by induction of HO-1 after treatment of animals with hemin [56]. Data of the present study reveal that incubation of macrophages with a CO donor $[Ru(CO)_3Cl_2]_2$ significantly reduced the cytotoxicity elicited by H_2O_2. Neither bilirubin, biliverdin, $FeSO_4$, nor $FeCl_3$ showed any effect on H_2O_2-induced cell death. This suggests that CO production may contribute to the antiapoptosis effect of HO-1 in macrophages. In related to the mechanism of CO inhibition of H_2O_2-induced cytotoxicity is still unclear. Ryter et al. indicated that CO protected oxidant-induced lung injury through a mechanism dependent on activation of the p38β/MKK3 pathway [57]. In addition, several intracellular proteins such as cytochrome p-450, cytochrome *c* oxidase, and NAD(P)H oxidase have been shown as direct physical targets of CO [58,59]. To elucidate the direct or indirect protective mechanism of CO against H_2O_2-induced apoptosis deserves scientific importance for further study.

Acknowledgements

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