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Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages

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Abstract

In the present study, we examine the protective mechanism of quercetin (QE) on oxidative stress-induced cytotoxic effect in RAW264.7 macrophages. Results of Western blotting show that QE but not its glycoside rutin (RUT) and quicitrin-induced HO-1 protein expression in a time- and dose-dependent manner, and HO-1 protein induced by QE was blocked by an addition of cycloheximide or actinomycin D. Induction of HO-1 gene expression by QE was accompanied by inducing ERKs, but not JNKs or p38, proteins phosphorylation. Addition of PD98059, but not SB203580 or SP600125, significantly attenuates QE-induced HO-1 protein and mRNA expression associated with blocking the expression of phosphorylated ERKs proteins. H_2O_2 addition reduces the viability of cells by MTT assay, and appearance of DNA ladders, hypodiploid cells, and an increase in intracellular peroxide level was detected. Addition of QE, but not QI or RUT, significantly reduced the cytotoxic effect induced by H_2O_2 associated with blocking the production of intracellular peroxide, DNA ladders, and hypodiploid cells. QE protection of cells from H_2O_2-induced apoptosis was significantly suppressed by adding HO inhibitor SnPP or ERKs inhibitor PD98059. Additionally, QE protects cells from H_2O_2-induced a decrease in the mitochondrial membrane potential and a release of cytochrome *c* from mitochondria to cytosol by DiOC6 and Western blotting assay, respectively. Activation of apoptotic proteins including the caspase 3, caspase 9, PARP, D4-GDI proteins was identified in H_2O_2-treated cells by Western blotting and enzyme activity assay, and that was significantly blocked by an addition of QE, but not RUT and QI. Furthermore, HO-1 catalytic metabolites carbon monoxide (CO), but not Fe^{2+}, Fe^{3+}, biliverdin or bilirubin, performed protective effect on cells from H_2O_2-induced cell death with an increase in HO-1 protein expression and ERKs protein phosphorylation. These data suggest that induction of HO-1 protein may participate in the protective mechanism of QE on oxidative stress (H_2O_2)-induced apoptosis, and reduction of intracellular ROS production and mitochondria dysfunction with blocking apoptotic events were involved. Differential anti-apoptotic effect between QE and its glycosides RUT and QI via distinct HO-1 protein induction was also delineated.
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Keywords: Flavonoids; Quercetin; Heme oxygenase 1; Reactive oxygen species; ERKs

1. Introduction

Reactive oxygen species (ROS) are generated under various physiological and pathological conditions such as inflammation, aging, and carcinogenesis [1–3]. An increase in intracellular ROS level has been shown to damage tissues and cells via lipid peroxidation, protein cross-linkage, and DNA breakage processes, which is partially prevented by anti-oxidants and free radical scavengers. Therefore, agents with ability to prevent

Abbreviations: HO-1, heme oxygenase 1; SnPP, tin protoporphyrin; CO, carbon monoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glutaldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; ERKs, extracellular regulatory kinases; MAPKs, mitogen activated protein kinases; JNKs, c-Jun N-terminal kinases

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ROS-induced injury may reserve potential to be further developed.

Some natural anti-oxidant products have been shown to protect cells from oxidative injury. Flavonoids are found in plants, and act as pharmacological active components in Chinese herbs. Multiple biological activities of flavonoids including vasodilatory, anti-inflammatory, anti-viral, anti-oxidant, and anti-carcinogenic effects have been identified [4–8], and the anti-oxidant activity of flavonoids has been given more attention. Flavonoids such as quercetin, catechin and kaempferol are better anti-oxidants than the anti-oxidants Vitamin C and Vitamin E [9]. Among them, quercetin is one of the most widely distributed flavonoids in plants, and several pharmacological effects such as suppression of cell proliferation, protection of LDL oxidation, prevention of platelet aggregation, and induction of apoptosis have been found [10,11]. The preventive effects of quercetin from apoptosis have been reported in several cells such as fibroblasts, cardiomyoblast cells, and epithelial cells [12,13]. Generally, quercetin was able to induce apoptosis in tumor cells through activation of caspase 3 cascade and suppression of heat shock protein 70 [14,15]. Yokoo and Kitamura [16] indicated that quercetin inhibition of apoptosis via blocking activator protein 1 (AP-1) activation. However, the relationship between HO-1 and quercetin prevention of apoptosis is still undefined.

Heme oxygenases (HOs) are enzymes responsible for catalyzing heme degradation, and four metabolites including iron, carbon monoxide (CO), biliverdin, and biliverdin have been identified. There are three types of HOs including HO-1, HO-2 and HO-3 were found. Among them, HO-1 is inducible and localized in the non-neural tissues in response to stressful conditions, whereas HO-2 and HO-3 are constitutively expressed and predominantly found in neural cells [17]. Recent evidences indicated that HO-1 played as a key role in defence mechanisms against oxidative damages [18,19]. Mice lacking functional HO-1 showed alternative iron metabolism and chronic inflammation, and an increased mortality after lipopolysaccharide (LPS) challenge was observed. Overexpression of HO-1 in cells resulted in a marked reduction in injury and cytotoxicity induced by oxidative stress [20,21]. In contrast, Dennery et al. [22] found that disruption of HO-1 was able to protect against hyperoxia via diminishing the generation of toxic reactive intermediates such as iron and H_2O_2 in the lung. Therefore, most of evidences supported that HO-1 participated in the protective mechanism of cells from oxidative damages, however it is still unclear if HO-1 is involved in flavonoids protection of cell death induced by oxidative stress.

Structural modifications have been shown to affect the biological activities of flavonoids. William et al. [23] indicated that OH substitutions were important in the anti-oxidant activities of flavonoids. The studies of structure-FPTase inhibitory activity indicated that the number, position and substitution of OH groups of the A and B rings of flavonoids, and unsaturation of the C2–C3 bond are important factors affecting inhibition on FPTase by flavonoids [24]. Park and Chiou [25] indicated that OH groups, below three or above four, produced no effects on the ocular blood flow. It appeared that three OH groups in the flavonoids were the best to increase the ocular blood flow. In addition to OH substitutions, glycoside addition is a common event in the metabolism of flavonoids in vivo. Regev-Shoshani et al. [26] indicated that glycosylation of polyphenols was able to inhibit protein oxidation, and maintain their anti-oxidant activity via extending their half-life in the cells. Our previous studies demonstrated that glycoside addition attenuated the apoptotic activities of flavonoids [4]. Additionally, we found that flavonoids without glycosides addition exhibited more significant inhibitory effects on LPS-induced NO and PGE_2 production than respective glycosylated flavonoids via HO-1 induction [8,27]. However, the effect of glycoside on flavonoids prevention of oxidative stress-induced damage is still unclear. Results of the present study show that quercetin, but not its respective glycosides quercitrin or rutin, possessed effective preventive ability on H_2O_2-induced apoptosis. The preventive mechanism involving activation of HO-1 gene expression, inhibition of caspases and mitochondrial cascade is delineated.

2. Materials and methods

2.1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC). 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) and maintained in a 37 °C humidified incubator containing 5% CO_2.

2.2. Agents

The structurally related flavonoids including quercetin, quercitrin and rutin were obtained from Sigma Chemical (St. Louis, MO). The chemicals including bilirubin, ferric (III) Chlroide ($FeCl_3$), ferrous (II) Sulfate ($FeSO_4$), tricarbonyldichlororuthenium (II) dimmer $[Ru(CO)_3Cl_2]_2$(RuCO), trichlororuthenium ($RuCl_3$), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), hydrogen peroxide (H_2O_2), tin protoporphyrin (SnPP), *N*-acetyl cysteine (NAC), actinomycin D, cycloheximide, 2′, 7′-dichlorodihydrofluorescein-diacetate (DCHF-DA) and propidium iodine (PI), 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) were obtained from Sigma Chemical. Biliverdin was purchased from ICN Biomedical (USA). The antibodies of anti-HO-1, anti-α-tubulin, anti-pERK, anti-pP38, and anti-pJNK, anti-

PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, SB203580, and SP600125 were obtained from USB Biotechnology.

2.3. Western blotting

Total cellular extracts were prepared according to our previous papers, separated on 8–12% SDS-polyacrylamide minigels, and transferred to immobilon polyvinylidenedifluoride 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. RT-PCR

Cells were treated with each of the QE and present of PD98059 or SB203580 or SP600125 for 6 h and then washed with ice-cold PBS. Total RNA was isolated using a total RNA extraction kit (Amersham Pharmacia, 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 the cDNA using the following sense and antisense primers, respectively, for HO-1: CTGTGTAACCTCTGCTGTTCC and CCACACTACCTGA-GTCTACC; and for GAPDH: TG-AAGGTCGGTGTGAACGGATTTGGC and CATGTA-GGCCATGAGGTCCACCAC. 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 PT-PCR beads (Amersham Biosciences, UK). The amplification sequence protocol was 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s for 30 cycles. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining [27].

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 phydro eroxides 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 QE, QI, RUT or NAC in the present of H_2O_2 for 2 h. 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 was detected using a 525-nm (FL1-H) band-pass filter by flow cytometric analysis [5].

2.6. Cell viability assay

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) was used as an indicator of cell viability as determined by its mitochrondrial-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 ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories). Data of cellular viability were expressed as the percentage of control (survival of control) in the present study.

2.7. LDH release assay

The percentage of LDH release was expressed as the proportion of LDH released into medium compared to the total amount of LDH present in cells treated with 2% tritox-100 treated in the cells. 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 ml 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 presolidified 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.

2.9. Flow cytometry analysis

Cells were treated with the indicated compounds for a further 12 h. Trypsinized cells were washed with ice-cold PBS and were in 70% ethanol at −20 °C for at least 1 h. After fixation, cells were washed twice, 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 PI for 10 min. Fluoresence emitted from the PI–DNA complex was quantified after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickinson, San Jose, CA).

2.10. Caspase 3/CPP32 activity 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.

2.11. Measurement of mitochondrial membrane potential

3,3′-Dihexyloxacarbocyanine iodide (DiOC6(3)), a lipophilic cationic cyanine dye that alters occur at the mitochondrial level and is widely used to determine of mitochondrial membrane potential. Cells were treated with QE, QI or RUT in the present or without 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.

2.12. Cytochrome c release from mitochondrial in RAW264.7 cells

Cells were treated with QE, QI or RUT in the present of H_2O_2 for 12 h and harvested by centrifugation at 3000 rpm for 5 min at 4 °C. The cells pellets were washed once with ice-cold PBS and resuspended with 5 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. The cells were homogenized and centrifuged at 1200 rpm for 10 min at 4 °C to supernatants and pellets. The supernatants were then centrifuged at 12,000 rpm for 15 min at 4 °C and the obtained supernatants were 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. 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.

3. Results

3.1. Quercetion but not its glycosides quercitrin and rutin induces HO-1 protein expression in RAW264.7 macrophages

The chemical structures of quercetin (QE), quercitrin (QI) and rutin (RUT) were shown in Fig. 1A. QI and RUT possess a rhamnose and a rutinose (glucose + rhamnose) at the C3 of QE, respectively. Results of Fig. 2A showed that QE (but not QI and RUT) at the dose of 50 μM time-dependently induced HO-1 protein in RAW264.7 macrophages. As the same part of experiment, QE, at the doses of 25, 50, and 100 μM, dose-dependently induced HO-1 protein expression (Fig. 2B). No significant HO-1 protein induction was detected in QI- or RUT-treated cells. Addition of actinomycin D (ActD; 1 and 10 μg/ml) or cycloheximide (CHX; 0.25 and 0.5 μg/ml) significantly inhibited HO-1 protein expression induced by QE (Fig. 2C). We further examine the cytotoxic effect of QE, QI, and RUT in RAW264.7 cells by MTT assay. Results of Fig. 2D indicated that QE at the dose of 25 and 50 μM exhibited no significant reduction in cellular viability, however a slight but significant cytotoxic effect was detected in QE (100 μM)-treated cells. None cytotoxic effect was observed in QI- or RUT-treated RAW264.7 cells. These data indicated that QE is an HO-1 inducer,

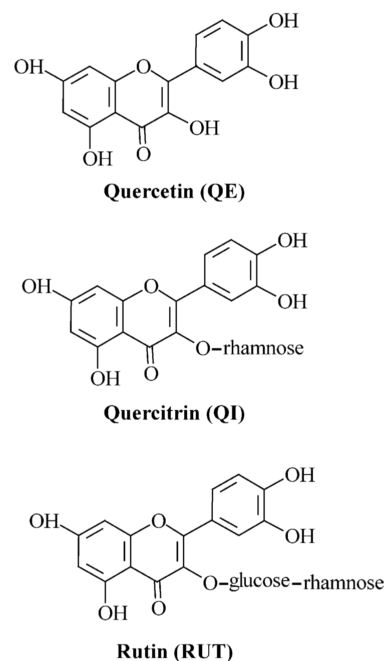

Fig. 1. Chemical structures of quercetin (QE), quercitrin (QI) and rutin (RUT).

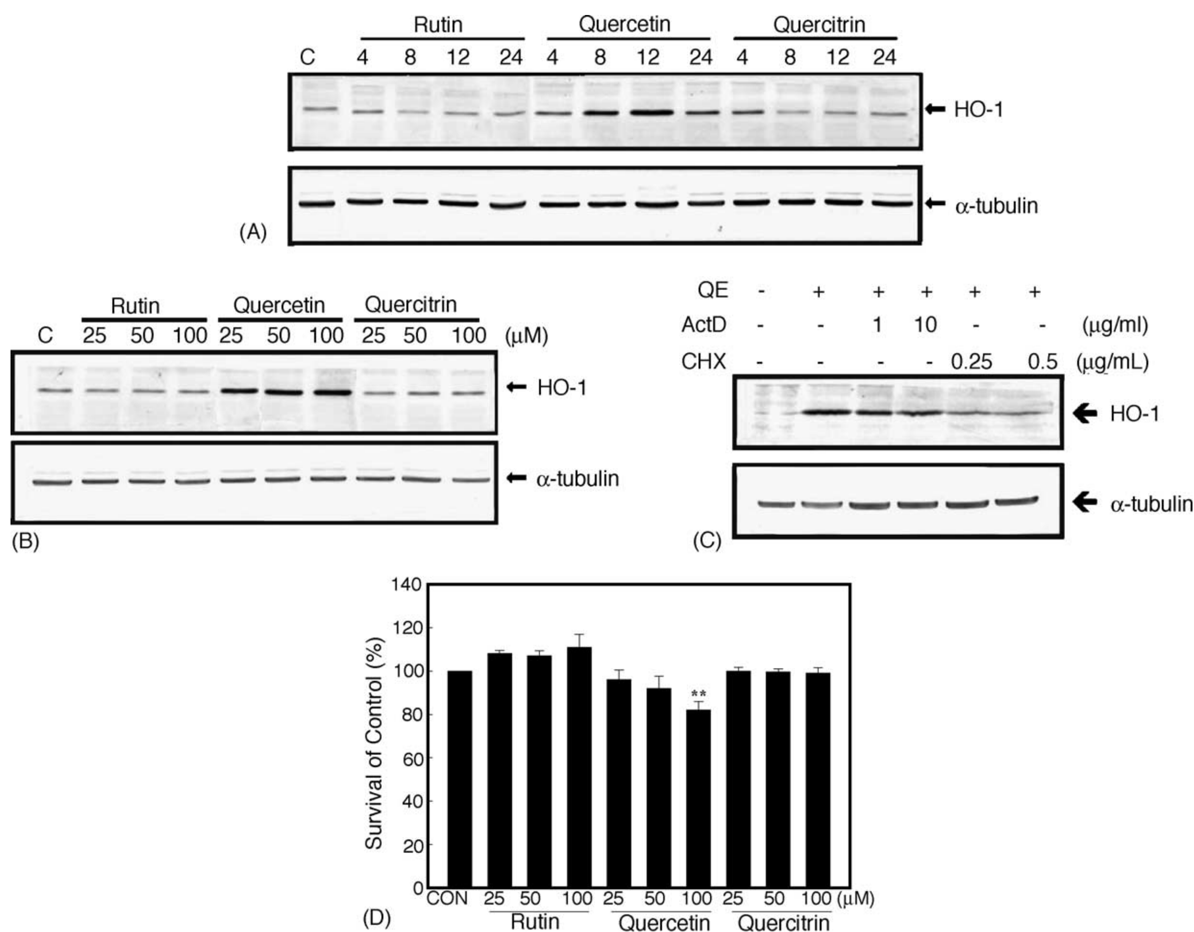

Fig. 2. Quercetin (QE), but not rutin and quercitrin, induction of HO-1 protein expression in RAW264.7 cells. (A) QE induction of HO-1 protein expression in a time-dependent manner. Cells were treated with rutin, quercetin, and quercitrin (50 μM) for 4, 8, 12, and 24 h, and expression of HO-1 protein was detected by Western blotting. (B) QE induction of HO-1 protein expression in a dose-dependent manner. Cells were treated with different concentrations (25, 50, and 100 μM) of rutin, quercetin, and quercitrin for 12 h, and the expression of HO-1 protein was analyzed. (C) Inhibition of QE-induced HO-1 protein expression by actinomycin D (ActD) or cycloheximide (CHX). Cells were treated with QE (50 μM) in the presence or absence of ActD (1 and 10 μg/ml) or CHX (0.25 and 0.5 μg/ml) for 12 h, and the expression of HO-1 protein was analyzed. (D) Examination of cytotoxic effect of rutin, quercetin, and quercitrin on RAW264.7 cells. Cells were treated with different concentrations (25, 50, and 100 μM) of rutin, quercetin, and quercitrin for 12 h. The viability of cells under different treatments was detected by MTT assay, and expressed as the percentage of control (survival of control). α-Tubulin was used as an internal control. C, control. Data are expressed as the mean ± S.E. $^{**}p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

and de novo protein synthesis is essential for QE induction of HO-1 protein.

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

We further investigate if activation of MAPKs cascades is involved in QE induction of HO-1 gene expression. RAW264.7 macrophages were treated with different doses of QE, QI or RUT for 40 min, and expression of phosphorylated MAPKs including ERKs, JNKs, and p38 proteins were examined by Western blotting using specific antibodies. Results of Fig. 3A show that QE (but not QI or RUT), at the doses of 25, 50, and 100 μM, induces the phosphorylation of ERKs, but not JNKs or p38, proteins in RAW264.7 cells. Pharmacological studies using specific inhibitors of MAPKs including PD98059 for blocking EKRs, SB203580 for blocking p38, and SP600125 for blocking JNKs, were performed in the study. Addition of PD98059 dose-dependently inhibits QE-induced ERKs protein phosphorylation, however neither SB203580 nor SP600125 exhibits effect in cells (Fig. 3B). And, PD98059 (but not SB203580 and SP600125), at the doses of 25 and 50 μM, significantly reduces the expression of HO-1 protein induced by QE (Fig. 3C). Results of RT-PCR using specific primers for HO-1 and GAPDH indicated that QE was able to induce HO-1 gene expression at mRNA level, which was inhibited by adding PD98059 (but not SB203580 and SP600125) (Fig. 3D). These data suggest that activation of ERKs locates at the upstream of HO-1 gene expression induced by QE.

3.3. QE protection of RAW264.7 cells from H_2O_2-induced cell death

We further examine the protective activity of QE, QI, and RUT on oxidative stress (H_2O_2)-induced cell death. Addition of H_2O_2 at the dose of 400 μM for 24 h decreased

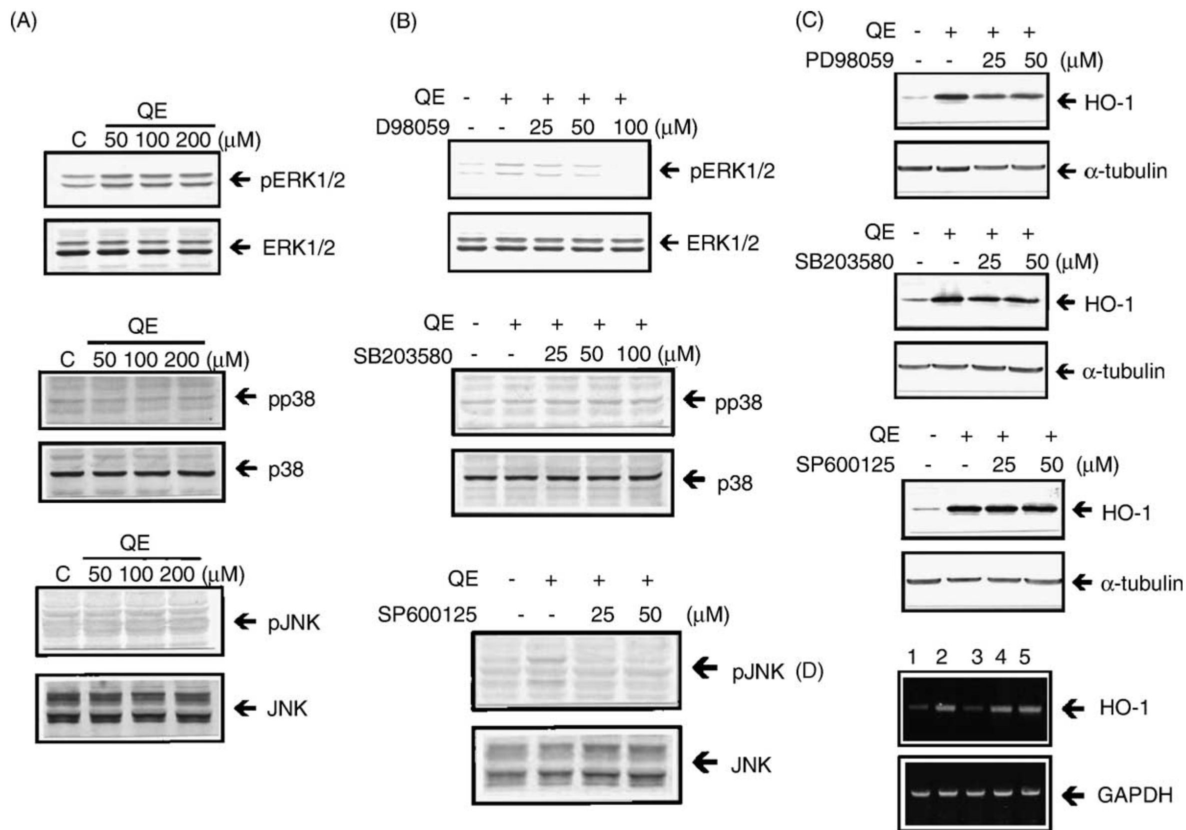

Fig. 3. Involvement of ERKs activation in QE induction of HO-1 gene expression. (A) QE induction of ERKs, but not p38 and JNKs, protein phosphorylation in RAW264.7 cells. Cells were treated with different concentrations (50, 100, and 200 μM) of QE for 40 min, and expression of phosphorylated and total ERKs/p38/JNKs protein was detected by Western blotting using specific antibodies. (B) PD98059, but not SB203580 and SP600125, inhibition of QE-induced ERKs (but not p38 and JNKs) proteins phosphorylation. Cells were pre-treated with or without PD98059, SB203580, and SP600125 (25, 50, and 100 μM) for 30 min followed by incubating with QE (50 μM) for a further 40 min. Expressions of phosphorylated and total ERKs (upper panel), p38 (middle panel) and JNKs (lower panel) proteins were analyzed by Western blotting using specific antibodies. (C) PD98059, but not SB203580 and SP600125, inhibition of QE-induced HO-1 protein expression in RAW264.7 cells. Cells were pre-treated with PD98059, SB203580, or SP600125QE (25 and 50 μM) for 30 min followed by incubating with QE (50 μM) for a further 12 h. The expression of HO-1 protein was analyzed by Western blotting. (D) PD98059 inhibition of QE-induced HO-1 mRNA expression. Cells were pre-treated with PD98059, SB203580, or SP600125QE (50 μM) for 30 min followed by incubating with QE (50 μM) for a further 6 h, and HO-1 mRNA level was analyzed by RT-PCR using specific primers. GAPDH was used as an internal control. 1, control; 2, QE; 3, QE + PD98059; 4, QE + SB203580; 5, QE + SP600125.

the viability of cells about 53% by MTT assay. Incubation of cells with different doses of QE, but not QI and RUT, (25 and 50 μM) with H_2O_2 (400 μM) showed significant protection on H_2O_2-induced cytotoxicity in RAW264.7 macrophages (Fig. 4A). In the condition of QE, QI or RUT pre-treatment for 6 h followed by H_2O_2 (400 μM) addition for a further 12 h, QE but not QI and RUT exhibited the activity to suppress the cytotoxicity induced by H_2O_2 (Fig. 4B). The protective effect of QE on H_2O_2-induced cell death was confirmed by LDH release assay as described in Fig. 4C. In order to examine if HO-1 involvement in QE protection of H_2O_2-induced cell death, an HO inhibitor tin protoporphyrin (SnPP) and ERKs inhibitor PD98059 were used in the study. As illustrated in Fig. 4D, neither SnPP nor PD98059 affects the viability of cells, and the protective effect of QE on H_2O_2-induced cell death was significantly attenuated by adding SnPP or PD98059 by MTT and the LDH release assays (Fig. 4D and data not shown).

3.4. QE inhibits H_2O_2-induced cytotoxicity through blocking apoptosis in macrophages

Both apoptosis and necrosis induced by H_2O_2 have been identified previously. Therefore, it is interesting to examine which type of cell death induced by H_2O_2 was prevented by QE. As illustrated in Fig. 5A, an increase in DNA ladders was detected in H_2O_2-treated cells, and that was inhibited by QE (but not QI and RUT) addition. Results of flow cytometry analysis showed that an increase in hypodiploid cells induced by H_2O_2 was blocked by QE (but not QI and RUT) addition (Fig. 5B and C). Results of Western blotting showed that induction of caspase 3 and caspase 9 protein processing, represented here is a decrease in pro-caspase 3 and pro-caspase 9 protein, with an increase in the cleaved fragment (85 kDa) of PARP and cleaved fragment (15 kDa) of D4-GDI was detected in H_2O_2-treated cells, which was significantly blocked by an addition of QE but not QI and RUT (Fig. 5D). Additionally, a colometric

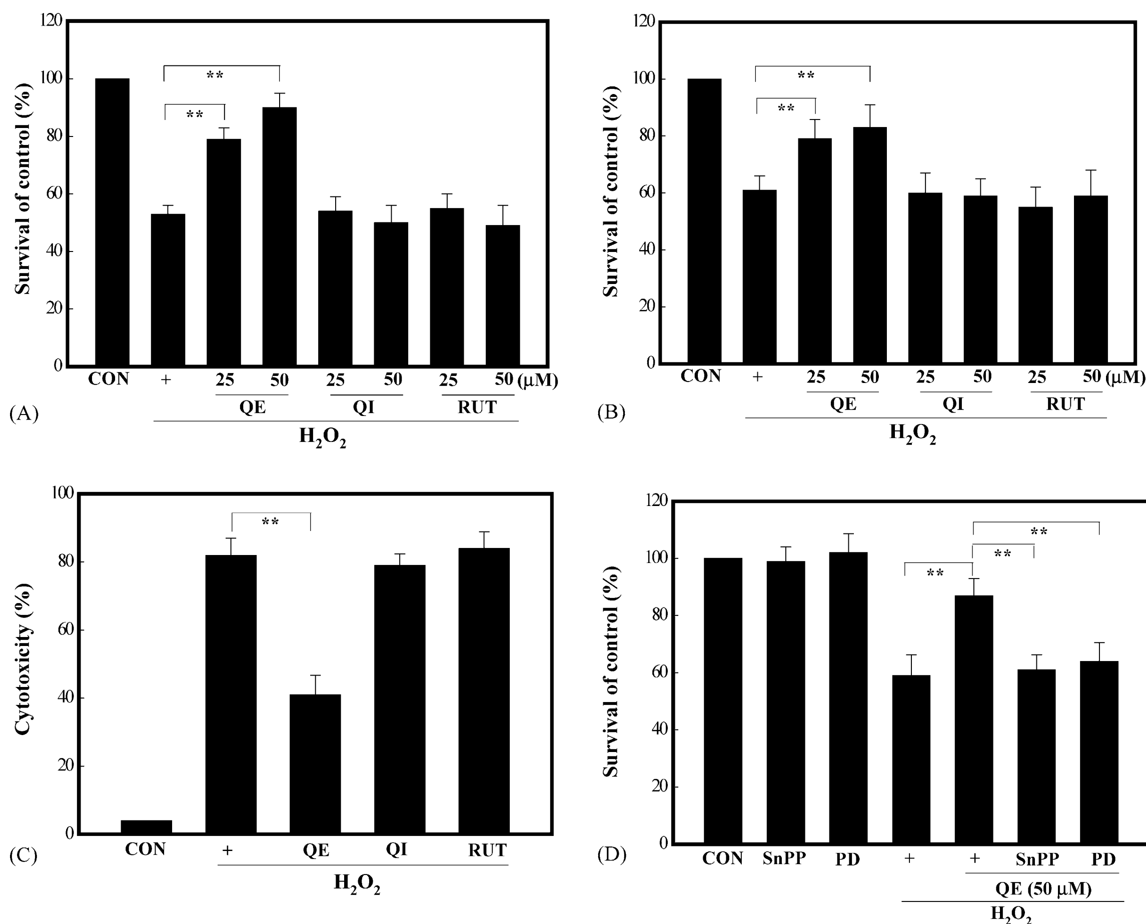

Fig. 4. QE protection of RAW264.7 cells from H_2O_2-induced cell death, which was attenuated by SnPP and PD98059 addition. (A) QE, (but not QI and RUT) inhibition of H_2O_2-induced cell death by MTT assay. Cells were treated with different concentrations (25 and 50 μM) of QE, QI, and RUT in the presence of H_2O_2 (400 μM) treatment for a further 12 h. The cellular viability was detected by MTT assay as described in Section 2. (B) Pretreatment of QE inhibits H_2O_2-induced cell death. Cells were pretreated with QE, QI, and RUT (25 and 50 μM) for 8 h followed by H_2O_2 (400 μM) treatment for a further 12 h. The cellular viability was detected by MTT assay. (C) QE inhibition of H_2O_2-induced LDH release in the culture medium. Cells were treated with QE, QI, and RUT (50 μM) in the presence of H_2O_2 for a further 12 h, and LDH released in medium was detected as described in Section 2. (D) Attenuation of QE protection of H_2O_2-induced cell death by addition of SnPP and PD98059. Cells were treated with QE (50 μM) in the presence or absence of SnPP (10 μM) or PD98059 (20 μM) followed by H_2O_2 treatment for a further 12 h. The viability of cells was detected by MTT assay. Data are expressed as the mean ± S.E. $^{**}p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

caspase 3-specific substrate Ac-DEVD-pNA was used to examine the activity of caspase 3 under different treatments. Results of Fig. 5E indicated that H_2O_2 induction of caspase 3 enzyme activity was detected in RAW264.7 cells, and the inductive caspase 3 activity was significantly blocked by QE (but not QI and RUT) (Fig. 5E). These data suggest that QE prevention of RAW264.7 cells from H_2O_2-induced cytotoxicity is via blocking the occurrence of apoptotic events.

3.5. QE reduction of intracellular ROS production and maintenance of mitochondrial membrane potential in H_2O_2-treated RAW264.7 cells

We further investigate the effect of QE on ROS production and mitochondrial membrane potential in the presence of H_2O_2 treatment. Results of Fig. 6A showed a representative of flow cytometry analysis using DCHF-DA as a fluorescent dye for ROS detection under different treatments in RAW264.7 cells, and data quantitated from three-independent experiments were described in Fig. 6B. It indicates that H_2O_2 addition induces an increase in intracellular peroxide level, which was significantly reduced by QE (but not QI and RUT) in RAW264.7 cells (Fig. 6). *N*-acetyl cysteine (NAC), a well-known ROS scavenger, blocking peroxide production induced by H_2O_2 was described as a positive control here. Additionally, we identified the effect of QE on mitochondrial function in H_2O_2-treated macrophages by flow cytometry analysis using $DiOC_6$ as a fluorescent dye. A decrease in mitochondrial membrane potential was observed in RAW264.7 cells under H_2O_2 treatment, and QE (but not QI and RUT) addition significantly attenuated H_2O_2-induced a loss in mitochondrial membrane potential (Fig. 7A). A release of cyt *c* from mitochondria to cytosol has been found in mitochondria-dependent apoptosis. Results of Fig. 7B indicate that addition of H_2O_2 induces the release of cyt *c* protein from mitochondria to cytosol, which was blocked

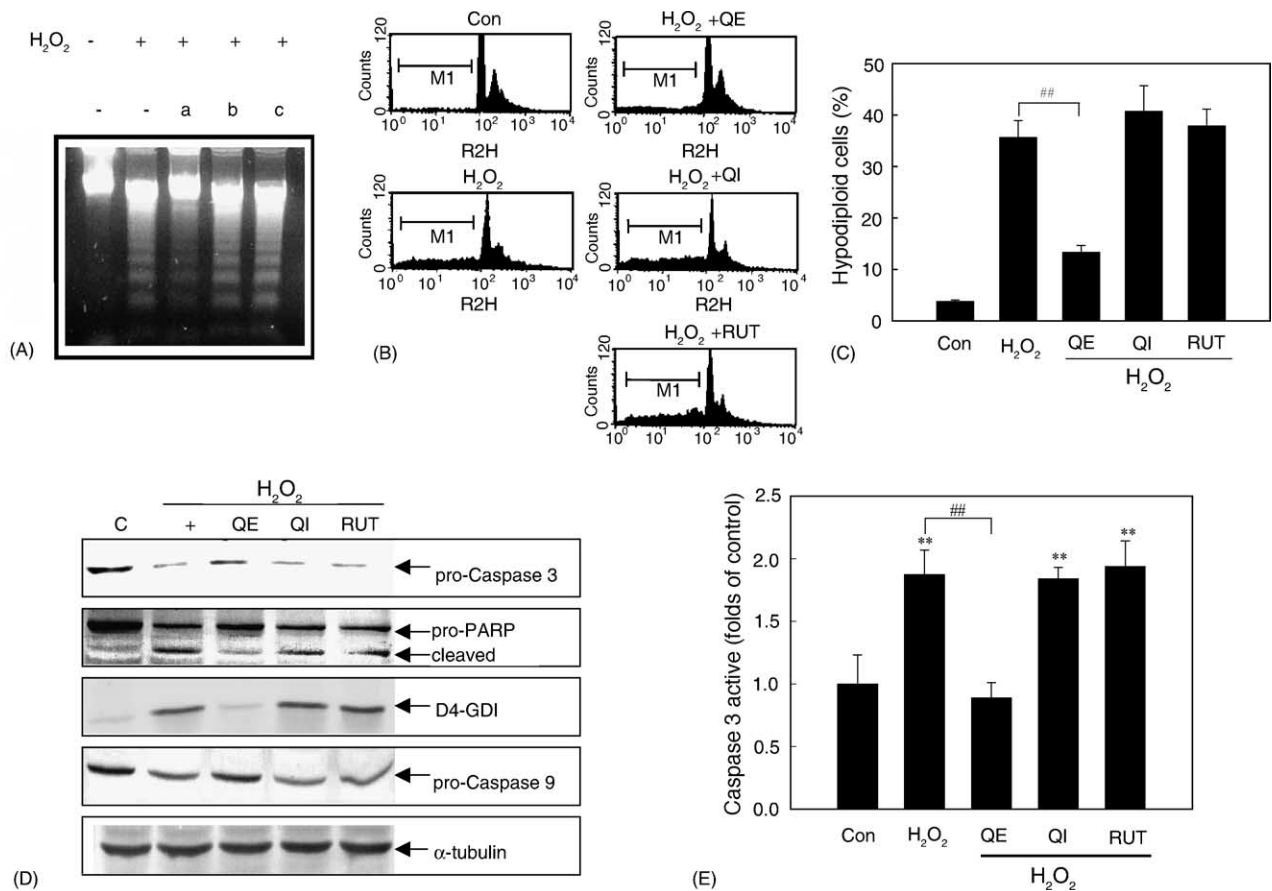

Fig. 5. QE, but not QI and RUT, decreased H_2O_2-induced apoptosis in RAW264.7 cells. (A) Cells were treated with QE (a), QI (b), and RUT (c) (50 μM) with or without H_2O_2 (400 μM) for 12 h. DNA integrity in cells was analyzed by agarose electrophoresis. (B) Cells were treated as described in (A), and the ratio of hypodiploid cells under different treatments was detected by flow cytometry analysis. A representative of data of flow cytometry analysis was presented. (C) The percentage of hypodiploid cells under different treatments was measured and quantitated from three-independent experiments. (D) Under the same condition in (A), the expression of caspase 3, caspase 9, PARP, D4-GDI, and α-tubulin protein was detected by Western blotting using specific antibodies. (E) Caspase 3 enzyme activity in cells under different treatments was measured using caspase 3-specific substrate Ac-DEVD-pNA. Each value is presented as the mean ± S.E. of three-independent experiments. $^{**}p < 0.01$ indicates a significant difference from the control. $^{\#\#}p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

by addition of QE (but not QI and RUT). These data indicate suppression of ROS production and a loss in mitochondiral membrane potential is involved in the QE prevention of H_2O_2-induced cell death.

3.6. Differential effect of HO metabolites on HO-1 induction and H_2O_2-induced cytotoxicity in RAW264.7 macrophages

Previous data indicated that induction of HO-1 protein might be involved in QE inhibition of cell death induced by H_2O_2, however the effect of HO metabolites on H_2O_2-induced cell death is still undefined. Five HO metabolites including $[Ru(CO)_3Cl_2]_2$ (CO, a CO donor), Fe^{2+}, Fe^{3+}, biliverdin, bilirubin were used to examine their effects on H_2O_2-induced cytotoxicity in macrophages. None of Fe^{2+}, Fe^{3+}, biliverdin and bilirubin performed effect on HO-1 protein expression and H_2O_2-induced cytotoxicity by Western blotting and MTT assay, respectively (Fig. 8A). In contrast, CO donor at the doses of 25, 50, 100 μM significantly induced HO-1 protein expression with an increase in the level of ERKs protein phosphorylation (Fig. 8B; upper and middle panels). Attenuation of H_2O_2-induced cytotoxicity by CO donor at the doses of 25, 50, 100 μM was also observed in RAW264.7 cells (Fig. 8B; lower panel).

4. Discussion

Results of the present study show that QE but not its respective glycosides RUT and QI prevents H_2O_2-induced apoptosis in macrophages, and suppression of both caspase 3 activation and decreasing mitochondrial membrane apoptotic cascades with an increase in HO-1 gene expression was identified in its preventive mechanism. Glycoside substitution playing as a negative moiety in the anti-apoptotic effect of flavonoids was identified in the present study.

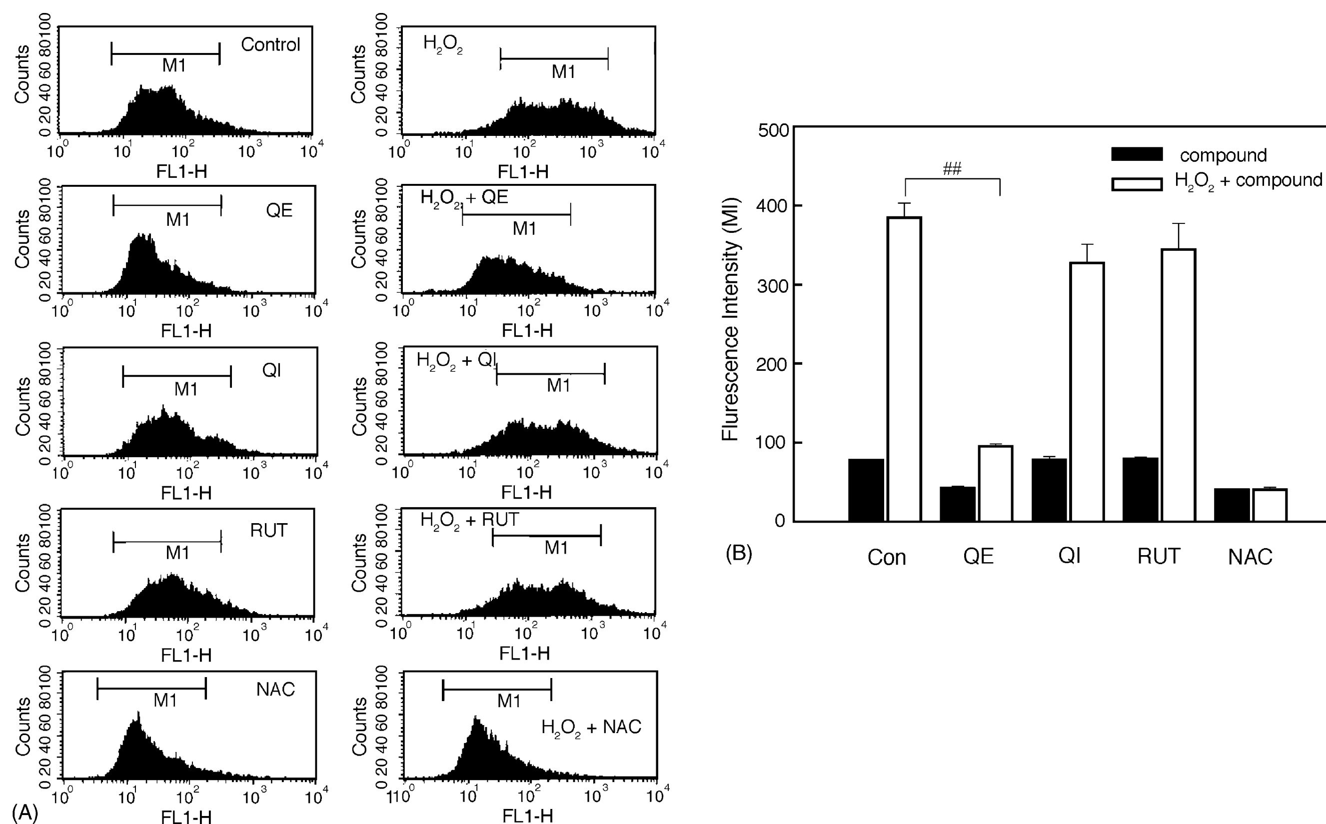

Fig. 6. QE (but not QI and RUT) reduces H_2O_2-induced intracellular peroxide level in RAW264.7 cells by DCHF-DA assay. RAW264.7 cells were treated with QE, QI, RUT (50 μM), and *N*-acetyl cysteine (NAC; 10 mM) in the presence or absence of H_2O_2 (400 μM) for 2 h. The level of intracellular peroxide in cells was measured by flow cytometry analysis using DCHF-DA as a fluorescence dye. (A) A representative of the data if flow cytometry analysis. (B) Data are derived and quantitated from three-independent experiments, and each value is presented as the mean ± S.E. $^{\#\#}p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

The protective effects of QE have been identified in different cells, however the mechanism is still unclear. Park et al. [28] indicated that QE was able to inhibit H_2O_2-induced apoptosis in H9c2 cells via blocking mitochondrial dysfunction. Ishikawa and Kitamura [29] indicated that the anti-apoptotic effect QE is by intervention in the JNK and ERK-mediated apoptotic pathways. Musonda and Chipman [30] indicated that the anti-oxidant potential might contribute to anti-carcinogenic and anti-inflammatory effects of QE. In contrast, QE also possessed ability to induce DNA damage via increasing intracellular H_2O_2 level, and induce DNA mutation in cells [31,32]. Therefore, the biological effects of QE in cells are controversial, and remain to be further elucidated. In the present study, we found that QE induced HO-1 gene expression via inducing ERKs phosphorylation, and inhibited H_2O_2-induced apoptosis in RAW264.7 cells. PD98059, an inhibitor of ERKs, attenuated the preventive effect of QE on H_2O_2-induced cytotoxicity with a decrease in HO-1 gene expression. Activation of caspase 3 cascade and a loss in mitochondrial membrane potential induced by H_2O_2 was significantly suppressed by QE. It suggests that ERK activation and HO-1 induction with blocking both caspase 3 cascade and a loss in mitochondrial membrane potential participate in QE prevention of H_2O_2-induced apoptosis.

Activation of intracellular kinase cascades has been shown in the regulation of HO-1 gene expression. Kietzmann et al. [33] showed that activation of JNK and p38 kinases was involved in induction of HO-1 gene expression in rat primary hepatocytes. Elbirt et al. [34] showed that HO-1 induced by arsenite was through activation of ERK and p38 in hepatoma cells. Our previous study showed that QE was an effective inducer of HO-1 gene [8] However, in related to the kinases involved in QE induction of HO-1 gene is still undefined. Here, we found that activation of ERKs but not JNK and p38 was identified in QE-treated macrophages, and attenuation of ERKs activation by PD98059 significantly reduced HO-1 protein expression induced by QE. It suggests that HO-1 gene induced by QE is through activation of ERKs but not JNK and p38 in RAW264.7 cells.

HO is the rate-limiting enzyme in the degradation of heme into bilirubin, carbon monoxide (CO), and free divalent iron (Fe^{2+}), and three isoforms have been identified. Among them, HO-1 is strongly induced by a variety of physiologic and pathophysiologic stimuli, including heme, heavy metals, cytokines, and nitric oxide. Accumulating evidence shows that the pivotal importance of HO-1 in mediating anti-oxidant, anti-inflammatory and anti-apoptotic effects. Choi et al. [35] indicated that over-expression

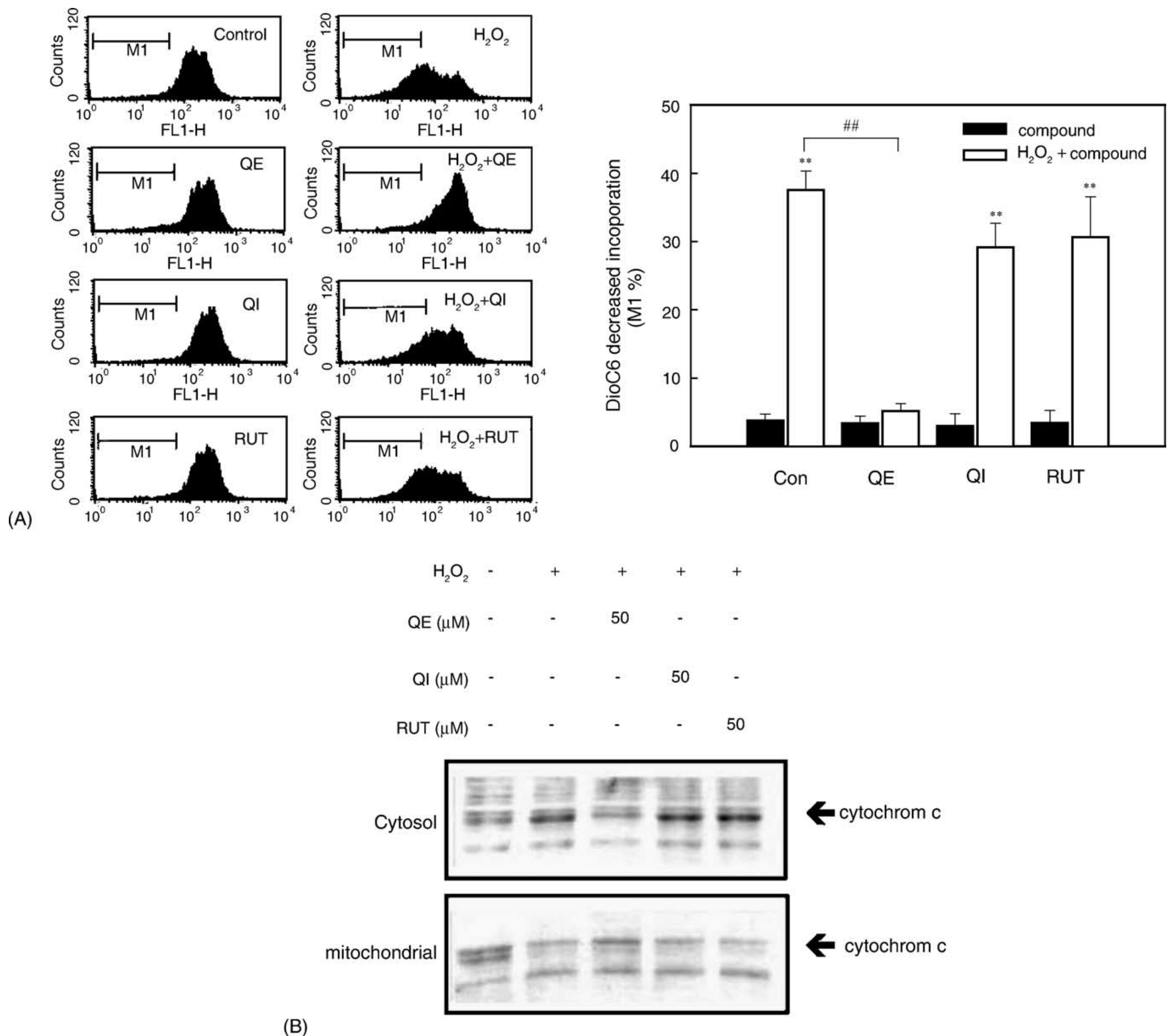

Fig. 7. QE (but not QI and RUT) protects RAW264.7 cells from H_2O_2-induced a loss of mitochondrial membrane potential and cytochrome *c* (cyt *c*) translocation. (A) Cells were treated with QE, QI, and RUT (50 μM) in the presence or absence of H_2O_2 (400 μM) for 6 h. The mitochondrial membrane potential of cells under different treatment was detected by flow cytometry analysis using DiOC6 as a fluorescence dye. Left panel, a representative of the data of flow cytometry analysis was presented. Right panel, ratio of M1 in different groups was quantitated from three-independent experiments. (B) QE (but not QI and RUT) at the concentration of 50 μM inhibited H_2O_2-induced cytochrome *c* release from mitochondria to cytosol. Cells were treated with of QE, QI, and RUT (50 μM) in the presence of H_2O_2 (400 μM) for 12 h. The expression of cytochrome *c* protein in the cytosolic and mitochondrial fractions was detected by Western blotting using specific antibody. $^{**}p < 0.01$ indicates a significant difference from the control. $^{\#\#}p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

of HO-1 inhibited FAS-induced apoptosis involving iron production. Vulapalli et al. [36] indicated that induction of HO-1 prevented IR-induced cardiac dysfunction and apoptosis. Braudeau et al. [37] indicated that HO-1 induction prolonged the survival of cardiac allograft. However, the role of HO-1 in flavonoids prevention of oxidative stress-induced apoptosis is still unclear. Results of the present showed that inhibition of HO-1 expression by PD98059 or HO-1 enzyme activity by SnPP significantly attenuated the preventive effect of QE on H_2O_2-induced apoptosis. It suggests that HO-1 may play a role in QE prevention of apoptosis induced by H_2O_2.

At least four metabolites produced by HO-1 including divalent iron, carbon monoxide (CO), biliverdin, and bilirubin have been reported. Several previous studies indicated that biliverdin and bilirubin were potent anti-oxidants, and possessed ability to inhibit ROS-induced DNA damages. Foresti et al. [38] reported that bilirubin induced by HO-1 decreased peroxynitrite-mediated cytotoxicity and reduction of postischemic myocardial dysfunction in rat heart. Stocker et al. [39] showed that bilirubin was able to scavenge peroxyl radicals in vitro and the anti-oxidant activity of bilirubin surpasses that of α-tocopherol. Additionally, CO has been shown to regulate vasocontriction/

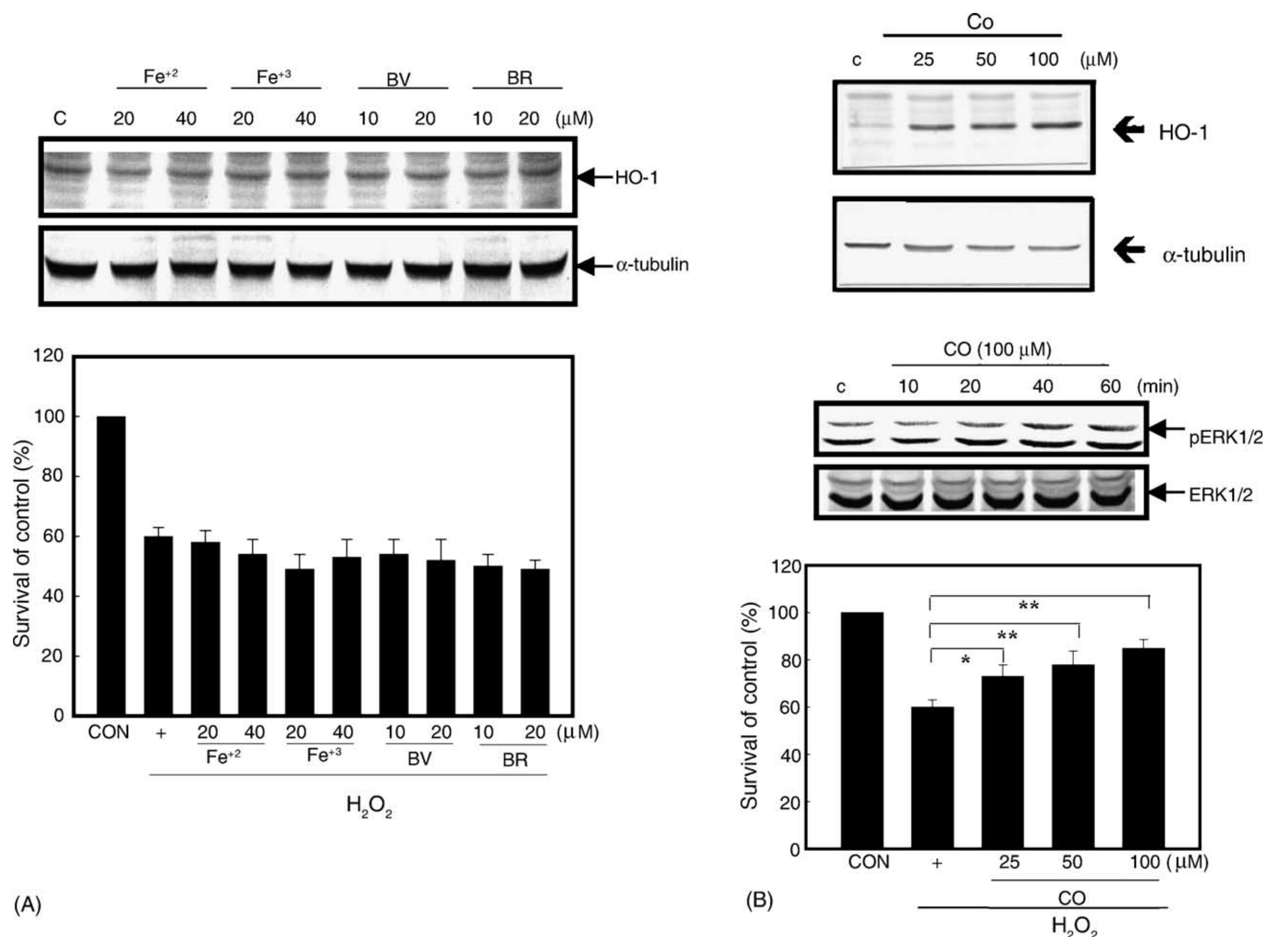

Fig. 8. CO (but not bilirubin, biliverdin, Fe^{2+}, Fe^{3+}) exhibits protective effect on H_2O_2-induced cell death with inducing HO-1 protein expression and ERKs protein phosphorylation in RAW264.7 cells. (A) Upper panel, cells were treated with $FeSO_4$ (Fe^{2+}; 20 and 40 μM), $FeCl_3$ (Fe^{3+}; 20 and 40 μM), biliverdin (BV; 10 and 20 μM), and bilirubin (BR; 10 and 20 μM) for 12 h, and expression of HO-1 protein was analyzed. Lower panel, cells were treated as described in ''Upper panel'' in the presence of H_2O_2 (400 μM) for 12 h. The viability of cells was detected by MTT assay. (B) CO induction of HO-1 protein expression and ERKs phosphorylation with a reduction in H_2O_2-induced cytotoxicity in macrophages. Upper panel, cells were treated with a CO donor $[Ru(CO)_3Cl_2]_2$ (25, 50 100 μM) for 12 h and expression of HO-1 protein was detected. Middle panel, cells were treated with $[Ru(CO)_3Cl_2]_2$ (100 μM) for different time points, and expression of phosphorylated (pERK1/2) and total ERKs (ERK1/2) protein was detected. Lower panel, cells were treated as in ''Upper panel'' in the presence of H_2O_2 (400 μM) treatment. The viability of cells was analyzed by MTT assay. Data are expressed as the mean ± S.E. of three-independent experiments. $^{**}p < 0.01$, indicates a significant difference from H_2O_2-treated group, as analyzed by Student's *t*-test.

vasprelaxation and production of proinflammatory molecules via activation of guanylyl cyclase/cyclic GMP (cGMP) and p38 mitogen-activated protein kinase (MAPK) in cells [40]. Otterbein et al. [41,42] indicated that exposure of low concentration of CO was able to increase the tolerance to hyperoxic lung injury in rats. Sato et al. [43] reported that CO suppressed graft rejection via inhibiting platelet aggregation, vascular thrombosis and myocardial infarction. Results of the present study appeared that CO, but not divalent ferric, bilirubin and biliverdin, effectively reduced H_2O_2-induced cell death in macrophages. It suggests that CO provides an important role in the cytoprotective effect of HO-1 induction.

Flavonoids have been shown to possess inhibitory activity on intracellular signal transduction process in response to chemical stimulus [44]. Johnson and Loo [45] reported that a lower concentration of quercetin inhibited oxidative stress-induced DNA, and a higher concentration of quercetin induced DNA damages via their proxidant activities. Kong et al. [46] also reported that a lower concentration of quercetin decreased the cell death via activating MAPKs, expressing survival genes (c-Fos, c-Jun) and defensive genes (phase II detoxifying enzymes; glutathione *S*-transferase, quinone reductase). Results of the present study show that QE at the doses below 100 μM significantly decreases H_2O_2-induced peroxide production by DCHF-DA assay without obvious cytotoxicity in cells, however QE induction of apoptosis is detected at the dose of 200 μM (data not shown). These data delineate a double-blade of QE, and show that lower doses of quercetin may contribute the cytoprotective effect in macrophages.

Although QE prevention of oxidative stress-induced cell death has been reported, results of the present provide the first evidence to indicate that HO-1 involves in the preventive mechanism of QE via activation of ERKs. Additionally, glycoside addition may as a negative moiety in QE

prevention of H_2O_2-induced apoptosis in RAW264.7 macrophages. It suggests that flavonoids with ability to induce HO-1 gene expression may reserve potential to protect oxidative damages for further applications.

Acknowledgements

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