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Quercetin protects against pulmonary oxidant stress via heme oxygenase-1 induction in lung epithelial cells

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ABSTRACT

The lung is a primary target for oxygen toxicity because of its constant exposure to high oxygen levels and environmental oxidants. Quercetin is one of the most commonly found dietary flavonoids, and it provides cytoprotective actions via activation of specific transcriptional factors and upregulation of endogenous defensive pathways. In the present study, we showed that quercetin increased the levels of heme oxygenase (HO)-1 expression and protected against hydrogen peroxide (H_2O_2)-induced cytotoxicity in lung epithelial cell lines. Quercetin suppressed H_2O_2-induced apoptotic events, including hypodiploid cells, activation of caspase 3 enzyme activity and lactate dehydrogenase release. This cytoprotective effect was attenuated by the addition of the HO inhibitor, tin protoporphyrin IX. In addition, the end products of heme metabolites catalyzed by HO-1, carbon monoxide and bilirubin, protect against H_2O_2-induced cytotoxicity in LA-4 cells. Quercetin may well be one of the promising substances to attenuate oxidative epithelial cell injury in lung inflammation.

1. Introduction

The airway and alveolar epithelium provide a direct interface between the environment and the internal milieu of the individual and they face the threat of oxidation from molecular oxygen. Reactive oxygen species (ROS) such as O_2^-, H_2O_2, OH, OCl−, are formed in the pulmonary epithelial and endothelial cells or released from activated macrophages and leukocytes as a result of normal biochemical processes, activation of inflammatory cells, and the damaging effects of inhaled gases and airborne particulates [1]. ROS damage the lungs and initiate cascades of pro-inflammatory reactions propagating pulmonary and systemic stress [2]. Pulmonary oxidant stress by overproduction of ROS plays an important pathogenetic role in disease conditions, including acute lung injury/acute respiratory distress syndrome (ALI/ARDS), hyperoxia, sepsis, radiation injury, and chronic obstructive pulmonary disease (COPD) [3].

Quercetin, a member of the flavonoid family, is one of the most prominent dietary antioxidants. It is ubiquitously present in foods including vegetables, fruit, tea and wine [4]. The preventive effects of quercetin from apoptosis have been reported in several kinds of cells such as macrophages [5], retinal pigmented epithelial cells [6], and glomerular mesangial cells [7]. Furthermore, quercetin has been recently reported to mediate cytoprotection through induction of heme oxygenase (HO)-1, which has a potent antioxidant property [8,9].

HO catalyzes the rate-limiting step in the degradation of heme to the bile pigments (i.e., biliverdin and bilirubin), carbon monoxide (CO), and iron. The inducible isoform HO-1 has an anti-apoptotic effect [10]. Fibroblasts overexpressing HO-1 are resistant to stress-mediated cell death [11]. Recent evidences have indicated

Abbreviations: ALI/ARDS, acute lung injury/acute respiratory distress syndrome; ARE, antioxidant response elements; CO, carbon monoxide; COPD, chronic obstructive pulmonary disease; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; HO, heme oxygenase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IPF, idiopathic pulmonary fibrosis; JNK, c-jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; Nrf2, nuclear factor E2-related protein 2; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pERK, phospho-ERK; PI, propidium iodide; pJNK, phospho-JNK; pp38, phospho-p38; RT-PCR, reverse transcription polymerase chain reaction; RuCO, $[Ru(CO)_3Cl_2]_2$; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SnPP, Tin protoporphyrin IX; WST-1, (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

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that HO-1 plays a key role in defence mechanisms against oxidative damages [12]. We have previously shown that quercetin exerted anti-allergic actions via activation of nuclear factor E2-related protein 2 (Nrf2)-HO-1 pathway in the mast cell [13]. Nrf2 is a noted cellular regulator of antioxidant and stress response because of its affinity for antioxidant response elements (ARE) [14]. Quercetin enhanced the ARE binding activity of Nrf2 and Nrf2-mediated transcription activity in human HepG2 cells [15]. Pharmacological research has suggested that mitogen-activated protein kinase (MAPK) is a central pathway involved in Nrf2 activation and translocation for highly specialized protein synthesis, including the most readily inducible HO-1 [16,17].

The purpose of this study was to determine whether quercetin could lead to an anti-apoptotic effect through induction of HO-1 expression against the oxidative stress on alveolar epithelial cells.

2. Materials and methods

2.1. Reagents

Quercetin, hemin, $[Ru(CO)_3Cl_2]_2$ (RuCO), bilirubin, and an anti-actin polyclonal antibody were obtained from Sigma Chemical (St. Louis, MO). Tin protoporphyrin IX (SnPP) was from Frontier Scientific (Carnforth, UK). Tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was from Roche (Basel, Switzerland). The anti-HO-1 antibody was from Stressgen Biotechnologies (Victoria, BC, Canada). The anti-Nrf2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK antibodies were from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

The murine alveolar epithelial cell line, LA-4 [18] was purchased from the American Type Culture Collection (Rockville, MD). LA-4 cells were maintained in DMEM supplemented with penicillin, streptomycin, amphotericin B, 1 mM sodium pyruvate, and 10% FCS.

2.3. Cell viability assay

LA-4 cells were incubated with or without quercetin, SnPP, RuCO, and bilirubin. Twelve hours after adding the appropriate concentration of H_2O_2, a colorimetric assay was performed based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. WST-1 (10%/well) was added to each well, and cells were incubated for 1 h. The plate was read at a wavelength of 450 nm with a reference at 650 nm.

2.4. Evaluation of cell damage

Cell damage was evaluated by dual staining with annexin V and propidium iodide (PI). LA-4 cells were pretreatment with or without quercetin for 1 h and stimulated with H_2O_2 for 24 h. Attached cells were pooled with any detached cells from the supernatant and annexin V and PI were added according to the manufacturer's instructions based on the method of Vermes et al. [19]. The stained cells were evaluated on a FACSCalibur flow cytometer using CELLQuest (Becton Dickinson, Franklin Lakes, NJ).

2.5. Lactate dehydrogenase (LDH) release assay

A colorimetric assay kit (Roche Applied Science, Indianapolis, IN) was used to quantify LDH released from cultured LA-4 cells into the surrounding culture medium according to the manufacturer's recommendations. Briefly, LA-4 cells were pretreated with quercetin for 1 h and stimulated with H_2O_2 for 12 h. The supernatant was added directly to equal volume of a reaction mixture consisting of catalyst/dye combination. After incubation at 25 °C for 15 min, absorbance was read at 490 nm with a reference wavelength at 690 nm.

2.6. Activities of caspase 3/CPP32 assay

The enzyme activities of caspase-3 were measured using a caspase colorimetric assay kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. In brief, LA-4 cells pretreatment with vehicle alone or quercetin for 1 h and stimulation with H_2O_2 for 12 h, then cytoplasmic protein (100 μg) extracted, and colorimetric substrate (5 μl) was added to 50 μl reaction buffer. After incubation for 1 h at 37 °C, absorbance at 405 nm was measured.

2.7. Quantitative real-time polymerase chain reaction (PCR)

Reverse-transcription (RT) and real-time PCR were performed as previously described [20]. Probes and primers to detect *Hmox1* and *Gapdh* were purchased from Nippon EGT (Toyama, Japan). Real-time PCR was performed in an ABI Prism7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). mRNAs of mouse HO-1 were indexed to the mouse GAPDH mRNA, using the following formula: $1/(2^{\Delta CT}) \times 100\%$.

2.8. Western blot analysis

Western blot analysis was performed as described previously [13]. For the analyses of HO-1 and MAPK, LA-4 cells were exposed to quercetin (0–60 μM) for indicated times. The total protein content was determined using the bicinchoninic acid (BCA) technique.

2.9. Immunocytochemical staining

Cells were cultured in LabTek chamber slides (Nalge Nunc International, Rochester, NY). Slides were fixed in Histochoice (Amresco, Solon, OH). Primary polyclonal antibodies against HO-1 or Nrf2 were incubated overnight at room temperature. For the detection of HO-1, an anti-rabbit IgG antibody, conjugated with horseradish peroxidase, was used as a secondary antibody. Slides were prepared with diaminobenzidine, and counterstained with methyl green. For the detection of Nrf2, an anti-rabbit IgG antibody conjugated with FITC was used as a secondary antibody, and PI was used for nuclear staining.

2.10. Statistical analysis

Data are expressed as the mean ± SD. Differences in measured variables between experimental and control groups were assessed using the Mann–Whitney *U* test. All analyses were performed using SPSS software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL).

3. Results

3.1. Quercetin protection against H_2O_2-induced cell death in LA-4 cells

First, we examined the protective effects of quercetin on H_2O_2-induced cytotoxicity in LA-4 cells. When LA-4 cells were preincubated with quercetin, the percentage of viable cells was increased by the concentration of quercetin in a dose-dependent manner: with 10, 30, 60 μM quercetin, 73%, 85%, 83%, were viable, respectively (Fig. 1A). With quercetin doses above 30 μM, cell viability reached plateau. Quercetin itself did not show any cytotoxicity

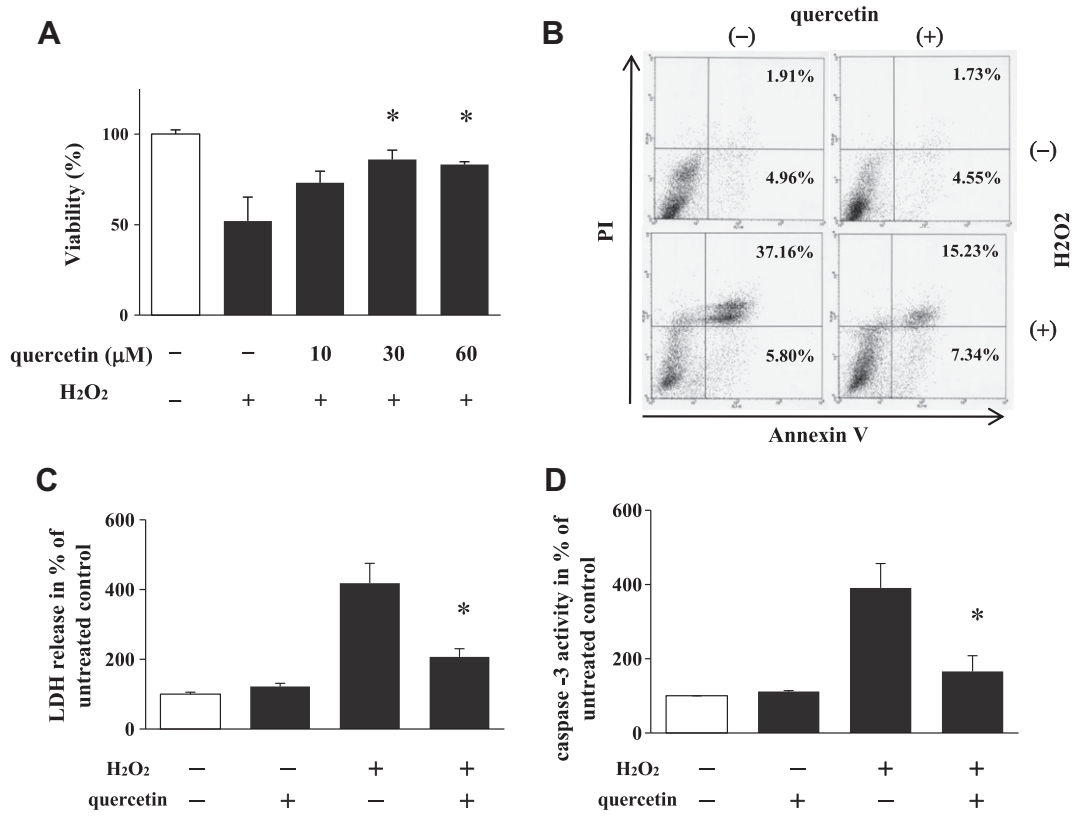

Fig. 1. Protective effect of quercetin from H_2O_2-induced cell damage. (A) LA-4 cells (1×10^4 cells/ well) were exposed to various concentrations (0, 10, 30, 60 μM) of quercetin for 1 h before exposure to 600 μM H_2O_2 for 12 h, cell viability was evaluated by WST-1 assay. (B) Dot plot of FITC-annexin V/PI flow cytometry. Flow cytometric analysis showed viable cells in lower left quadrant, early apoptosis in lower right quadrant and late apoptosis or necrosis in upper right quadrant. LA-4 cells (2.5×10^5 cells/ well) were cultured for 24 h with 600 μM H_2O_2 following 1 h pretreatment of vehicle alone or 60 μM quercetin. (C) LDH release assay. The culture supernatants, which were carefully removed and centrifuged, were added directly to an equal volume of a reaction mixture consisting of catalyst/dye combination. After incubation at 25 °C for 15 min, the absorbance was measured colorimetrically at 490 nm. (D) The enzyme activities of caspase-3. Cytoplasmic protein extracted from LA-4 cells and colorimetric substrate was added. After incubation for 1 h at 37 °C, the absorbance was measured colorimetrically at 405 nm. Each column represents the mean ± S.D. of more than three independent experiments.* $P < 0.05$ compared with only H_2O_2-treatment cell.

under our experimental conditions (data not shown). These results suggested that quercetin enhanced viability against H_2O_2-induced cytotoxicity.

3.2. Quercetin reduction of H_2O_2-induced apoptosis and necrosis

To evaluate the protective effects of quercetin, we examined the specific mode of cell death (apoptosis or necrosis) by dual staining with annexin V-FITC and PI. Quercetin reduced the cell population of H_2O_2-induced necrosis or late phase apoptosis from 37% to 15% (Fig. 1B). The protective effect of quercetin from H_2O_2-induced cytotoxicity was also identified by LDH release assay and caspase-3 activity assay. Both LDH release and caspase-3 activity decreased approximately by half on preincubation with quercetin (Fig. 1C and D). These results showed that the mode of cell death induced by H_2O_2 was mainly necrosis or late phase apoptosis and that quercetin prevented the induction of the necrosis and apoptosis by attenuation of the LDH release and the caspase-3 activity.

3.3. Quercetin induction of HO-1 expression in LA-4 cells

We next examined expression levels of HO-1 in LA-4 cells cultured with quercetin by quantitative real-time RT-PCR and Western blot analysis. Fig. 2A and B showed that the expression levels of HO-1 mRNA and protein increased in a concentration-dependent manner. The HO-1 mRNA expression levels were increased after 3 h exposure to quercetin (Fig. 2C). HO-1 protein expression levels determined showed that a small amount of HO-1 protein was expressed constitutively and an increase in its levels were readily observed after 6 h and continued during 24 h exposure to quercetin (Fig. 2D). Therefore, we used quercetin at the concentration of 60 μM in the following experiments. Consistent with the results observed in Western blot analysis, significant upregulation of HO-1 expression was observed immunohistochemically in the cytoplasm of LA-4 cells after exposure to quercetin, as compared with that of control (Fig. 2E). These results suggested that quercetin induced HO-1 in both levels of mRNA and protein.

3.4. Protective effects of quercetin-induced HO-1 activities on H_2O_2-induced cell death in LA-4 cells

We examined whether the significant amount of HO-1 induced by quercetin was involved in the protective effect against H_2O_2-induced cytotoxicity. For this purpose, we exposed LA-4 cells to SnPP, a well-known HO enzyme activity inhibitor, for 1 h with quercetin. Cell viability was evaluated by WST-1 assay. The protective effect of quercetin was inhibited by SnPP (Fig. 3A), indicating that quercetin protects against H_2O_2-induced cytotoxicity through HO activity.

Next, we investigated whether the metabolites of heme catabolism, CO and bilirubin participated in the protective effect of HO-1 against H_2O_2-induced cell death. LA-4 cells were treated with CO donor, RuCO, and bilirubin for 1 h. Fig. 3B showed that RuCO and bilirubin protected significantly from H_2O_2-induced cell death with increasing viability 28% and 19%, respectively. These results indicated that the metabolites of heme catabolism, CO and bilirubin,

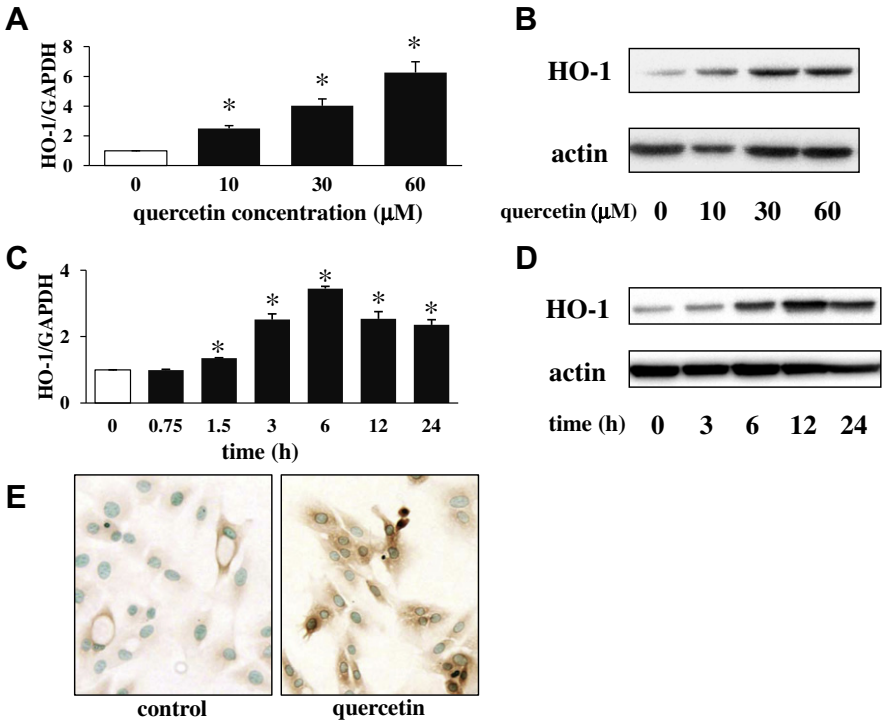

Fig. 2. Expression levels of HO-1 mRNA and protein. LA-4 cells were treated with various concentrations (0, 10, 30, 60 μM) of quercetin for the indicated times (0, 0.75, 1.5, 3, 6, 12, 24 h). Expressions of HO-1 mRNA and protein were determined by quantitative real-time RT-PCR (A and C), Western blotting (B and D) and immunohistochemistry (E). The results shown are representative of three independent experiments. $^{*}P < 0.05$ compared with non-treatment cell.

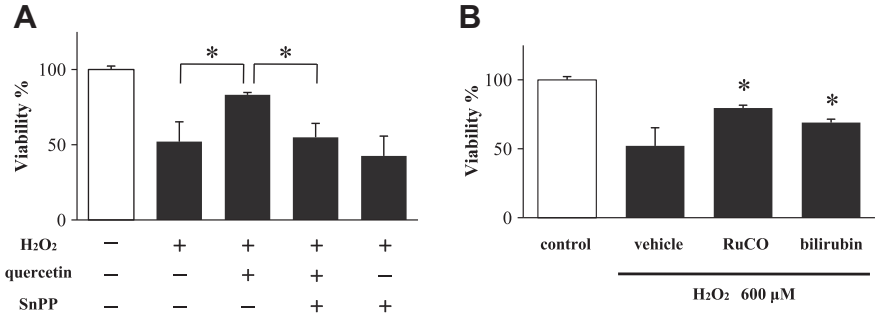

Fig. 3. Effect of SnPP and HO-1 metabolites (RuCO, bilirubin) on H_2O_2-induced cell injury. (A) Cells were exposed to quercetin (60 μM) with SnPP (75 μM) at the same time, the protective effect of quercetin against H_2O_2-induced cell injury was assessed by WST-1 assay. $^{*}P < 0.05$ indicates a significant difference between the designated groups. (B) The effects of RuCO (50 μM) and bilirubin (40 μM) on H_2O_2-induced cell death by WST-1 assay. Cells were exposed to RuCO or bilirubin with H_2O_2 at the same time. $^{*}P < 0.05$ compared with only H_2O_2-treatment cell.

were involved in the protective effects of quercetin on H_2O_2-induced cell death in LA-4 cells.

3.5. Involvement of MAPK signaling pathway in quercetin-induced protective effects

We next determined the role of MAPKs in quercetin-induced protective effects. As shown in Fig. 4A, phosphorylated JNK and ERK were detected after exposure to quercetin. However, no remarkable changes were observed in the phosphorylated levels of p38. These results indicated that quercetin was involved in the activation of ERK and JNK signaling pathway in LA-4 cells.

3.6. Translocation of Nrf2 from cytoplasm to nuclei in LA-4 cells by quercetin

The HO-1 gene is primarily regulated at the transcriptional level, and its inducibility is linked to the transcription factor Nrf2 [14]. Since Nrf2 reportedly translocates from cytoplasm to nucleus when Nrf2 is activated, we examined the localization of Nrf2 in LA-4 cells treated with quercetin. As shown in Fig. 4B, an increased nuclear localization of endogenous Nrf2 was observed in quercetin-treated cells after 4 h stimulation. These results suggested the possibility that quercetin leads Nrf2, which was involved in the induction of HO-1, from cytoplasm to nuclei accumulation.

4. Discussion

In this study, we demonstrated for the first time that quercetin was able to protect the mouse lung epithelial cell line, LA-4, from H_2O_2-induced cell death and that quercetin-induced cytoprotective effects were found to be due to an induction of HO-1 expression, with Nrf2 nuclear translocation.

In normal situations, the endogenous antioxidant network provides sufficient protection against reactive species such as ROS [21]. Oxidative stress, which may result in increased oxidative

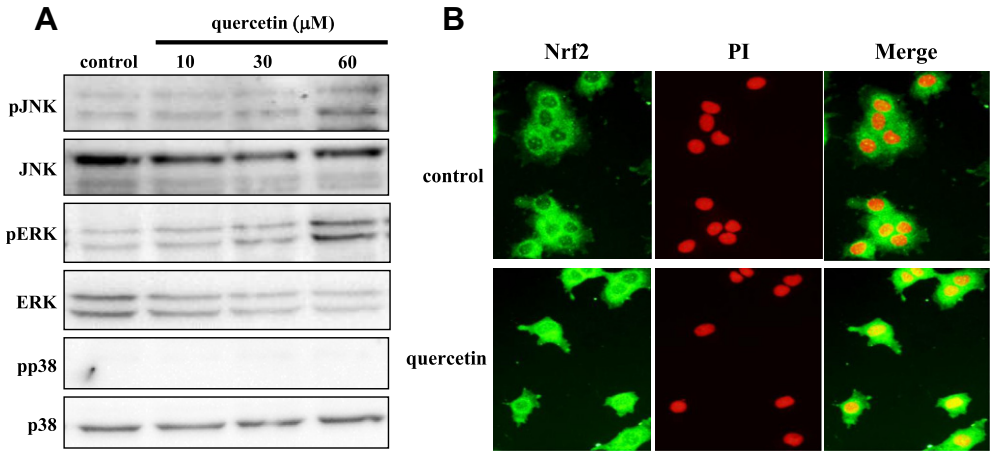

Fig. 4. Phosphorylation of MAPKs and localization of Nrf2 by quercetin. (A) Lysates from LA-4 cells incubated in the absence (control) or presence of quercetin (10, 30, or 60 μM) for 1 h were subjected to Western analysis using phospho-specific antibodies (JNK, pJNK, ERK, pERK, p38, pp38). (B) LA-4 cells were treated with 60 μM quercetin for 4 h and then subjected to immunohistochemical staining with antibody-specific Nrf2 followed by incubation with FITC-conjugated secondary antibodies. For each condition, an image of the cell nucleus stained with PI (red) specific for DNA. Nuclear localization of Nrf2 protein is indicated by the presence of yellow in the merged images.

damage, can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant defense system [22]. The lung is one of the organ systems most susceptible to oxidative damage, and epithelial cell injury is important in the pathogenesis of many pulmonary diseases, including ALI/ARDS and idiopathic pulmonary fibrosis (IPF). The protective effects of HO-1 against hyperoxia-induced lung cell injury and cell death have been studied *in vitro*. For example, HO-1 overexpression protected A549, a human alveolar epithelial cell line, against cell death induced by exposure to hyperoxia [23]. Increased production of ROS by activated neutrophils and decreased antioxidant capacity have been suggested to play a central role in the pathogenesis of ARDS [24]. Several evidences suggest that severe oxidative stress leading to tissue damage contributes to the high associated mortality rate [25–27]. Mortality of ARDS patients did not decrease between 1994 and 2006 [28], and it is important to establish a therapy for ARDS.

Many actions have been reported in bilirubin and CO, as HO-mediated degradation products of heme [29,30]. In the present study, we showed that the addition of the HO-1 inhibitor, SnPP, reduced the protective effect of quercetin against H_2O_2-induced lung epithelial cell death, and that exogenous RuCO and bilirubin prevented lung epithelial cells from H_2O_2-induced cell death. These results suggested that HO-1 plays an important role in the protective effects by flavonoids against oxidative stress.

However, some polyphenolic compounds including quercetin have been reported readily to oxidize in cell culture media by generating H_2O_2, and some products in cell culture media such as pyruvate and α-ketoglutarate was reported as a scavenger of H_2O_2 [31–33]. In this study, quercetin itself did not show any cytotoxicity, therefore, the generation of H_2O_2 by quercetin might not have the significant effect on the cell viability under our experimental conditions. Our media using in our experiments usually contain pyruvate. Although we did not measure the concentration of H_2O_2, pyruvate in our media might abolish or almost completely decrease the effects of H_2O_2 produced by quercetin. Of course, some remaining pyruvate might scavenge H_2O_2 added, because we have to use high concentration of H_2O_2 (600 μM) to optimize the effect of H_2O_2, and under this concentration of H_2O_2, we could exclude the effect of other oxidation products in cell culture media.

To investigate the role of MAPKs in quercetin-induced protective effects, we evaluated the phosphorylation of JNK, ERK, and p38. Quercetin induced the phosphorylation of JNK and ERK, but not p38. The effects of quercetin on signal transduction pathways were reported to vary dependent on cell types. For example, although quercetin facilitates the apoptosis of tumor cells such as HepG2 human hepatoma cells [34], it may in fact inhibit apoptosis in some nontumorigenic cells. Furthermore, Ishikawa and Kitamura showed that quercetin attenuated hydrogen peroxide-induced apoptosis in cultured mesangial cells by suppressing the activation of JNK and ERKs, but not p38 MAPK [35]. Their observations were in conflict with our results, suggesting that quercetin exerts its inhibitory effects through a different mechanism on signal transduction pathways.

Recently, a study regarding the tissue distribution of quercetin in rats has shown that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites is found in rat lungs [36]. Although HO-1 protein has reported to be elevated in the lungs of patients with ARDS [37], we could prevent lung oxidative damage in ALI/ARDS by increasing the amount of HO-1 protein induced by quercetin.

In the present study, we demonstrated that quercetin protected against H_2O_2-induced apoptosis and necrosis in lung epithelium through induction of HO-1. Furthermore, these protective effects of quercetin were mediated by an HO-1 dependent pathway. Considering that HO-1 is one of several molecules emerging as a central player in protecting against cellular damage, quercetin or its derivatives will be the key to new therapeutic strategies for ameliorating the lung injury induced by oxidant stress through HO-1 induction.

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