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Naringenin Exerts Cytoprotective Effect Against Paraquat-Induced Toxicity in Human Bronchial Epithelial BEAS-2B Cells Through NRF2 Activation

Biswajit Podder, Ho-Yeon Song, and Yong-Sik Kim*

Department of Microbiology, School of Medicine, Soonchunhyang University, Cheonan 330-090, Republic of Korea

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**Corresponding author*
Phone: +82-41-570-2413;
Fax: +82-41-577-2415;
E-mail: yongsikkim@sch.ac.kr

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We have previously shown that paraquat (PQ)-induced oxidative stress causes dramatic damage in various human cell lines. Naringenin (NG) is an active flavanone, which has been reported to have beneficial bioactivities, including antioxidative, anti-inflammatory, and antitumorigenic activities, with a relatively low toxicity to normal cells. In this study, we intended to assess the cytoprotective effect of NG against PQ-induced toxicity in the human bronchial epithelial BEAS-2B cell line. Co-treatment with NG in PQ-treated BEAS-2B cells can reduce PQ-induced cellular toxicity. NG can also decrease the generation of intracellular ROS caused by PQ treatment. We also observed that treatment with NG in PQ-exposed BEAS-2B cells can significantly induce the expression of antioxidant-related genes, including *GPX2*, *GPX3*, *GPX5*, and *GPX7*. NG co-treatment can also activate the NRF2 transcription factor and promote its nuclear translocation. In addition, NG co-treatment can induce the expression of NRF2-downstream target genes such as that of heme oxygenase-1 *(HO-1)* and NAD(P)H:quinone oxidoreductase 1 *(NQO1)*. A small interfering RNA study revealed that the knockdown of *NRF2* can abrogate NG-mediated protection of the cells from PQ-induced cellular toxicity. We propose that NG effectively alleviates PQ-induced cytotoxicity in human bronchial epithelial BEAS-2B cells through the NRF2-regulated antioxidant defense pathway, and NG might be a good therapeutic candidate molecule in oxidative stress-related diseases.

Keywords: Naringenin, paraquat, antioxidant-related genes, human bronchial epithelial BEAS-2B cells

Introduction

Paraquat (PQ; 1, 1′-dimethyl-4,4′-bipyridinium dichloride), a strong herbicide, has been extensively used in the agricultural field and has also been used as a experimental reactive oxygen species (ROS) generator to assess ROS-induced organ injury, such as pulmonary, renal, and neural toxicities [1, 24]. It has been known that the lung is the principal organ to be affected by PQ intoxication. Inhibition of PQ-induced injury may provide new opportunities for the prevention and treatment of PQ-induced diseases [10, 15, 19].

A balance of intracellular oxidation and reduction is essential for maintaining cellular functions, and the development of intracellular ROS imbalance causes various diseases, including cancer, diabetes, myocardial dysfunction, and renal injury [1, 2]. In previous studies, antioxidant therapies have been shown to exert protective effects against PQ-induced toxicity [14, 19].

Many traditional medicines and phytochemicals have been considered as potential therapeutic candidates for the management of intracellular oxidative balance owing to their decreased cytotoxicities and potent pharmacological features [25]. Naringenin (NG; 4,5,7-trihydroxyflavone) is the aglycone form of naringin, one of the naturally occurring flavanones. NG is predominantly found in citrus fruits, tomatoes, cherries, and grape fruits [13]. NG has shown a variety of pharmacological functions such as anti-

inflammatory, antioxidant, and anticancer activities in various human diseases, including cancer [5, 18, 22]. In previous studies, we have demonstrated that various phytochemicals have a significant cytoprotective effect on PQ-induced ROS stress in various cell lines [14, 19, 26]. However, the mechanism of the efficacy of NG against PQ-induced cellular toxicity in human bronchial epithelial BEAS-2B cells remains obscure.

The nuclear factor erythroid 2-related factor 2 (*NRF2*) acts as the master regulator of antioxidant-related genes such as those of heme oxygenase 1 *(HO-1)* and NAD(P)H quinone oxidoreductase 1 *(NQO1)* through binding to antioxidant response element (ARE) on the target promoter regions [17, 23]. Lack of NRF2 expression in animal models has high susceptibility to a range of chemical toxicity and diseases [4, 16]. The overexpression of NRF2 provides an advantage against cell toxicity. The protective role of NRF2 against PQ-induced cell toxicity has been well elucidated and confirmed by performing siRNA methodologies [10, 14].

However, to the best of our knowledge, no reports have documented the precise biological action of NG against PQ intoxication in human bronchial epithelial BEAS-2B cells. Therefore, this study was conducted to provide the first evidence of the protective effect of NG through the modulation of NRF2-driven, ARE-mediated gene expression *in vitro*.

Materials and Methods

Cell Culture and Treatment

BEAS-2B, a human bronchial epithelial cell line (#CRL-9609), was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained in bronchial epithelial cell basal medium (BEBM) containing 1% penicillin/streptomycin. Cells were grown to 70–80% confluency before performing the experiments. Cells were co-treated with various concentrations of NG, ranging from 5 to 100 μM, and 0.2 mM PQ in the culture medium.

Reagents

The culture medium BEBM was obtained from Lonza (Walkersville, MD, USA). Naringenin, paraquat, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), trypan blue stain solution, Triton-X100, and BAY11-7082 ((E)-3-[(4-methylphenylsulfonyl]-2-propenenitrile) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA) and antibiotics/antimycotics solutions (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) were obtained from Gibco (Grand Island, NY, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (D2DCF-DA) reagent was purchased from Invitrogen (Carlsbad, CA, USA). A lactate dehydrogenase (LDH) assay kit was supplied by Roche (Pleasanton, CA, USA). Primary antibodies against NRF2, HO-1, NQO1, and Lamin B, and relevant secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody for β-actin was obtained from Abcam (Cambridge, MA, USA).

Cell Viability and Morphology Analysis

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone [7]. Briefly, 1×10^5 BEAS-2B cells/ml were plated in a 96-well plate containing a final volume of 200 μl and incubated for 24 h to allow the cells to reach 80% confluency. Cells were co-treated with different concentrations of NG and PQ (0.2 mM) and viability was measured at different time periods. After incubation for the indicated time points, 20 μl of MTT (5 mg/ml) was added to each well and intercellular reduction of soluble yellow MTT into insoluble purple formazan crystal was allowed to proceed. The supernatant was then removed, and the formazan crystal was dissolved in 100 μl of DMSO. The plate was then incubated for another 30 min and the absorbance was measured at 590 nm using a Victor X3 multilabel reader (Perkin Elmer, Waltham, MA, USA). Data on cellular viability are shown as the percentage of control (survival percentage of control).

LDH Release Assay

LDH activity assays were performed in BEAS-2B cells using a colorimetric technique. First, 1×10^5 cells were seeded in 96-well plates and grown to 70–80% confluency. The cells were then co-treated with various concentrations of NG and PQ (0.2 mM). After treatment, the LDH assay was performed according to the manufacturer's protocol. Briefly, 100 μl of the cell culture supernatant was transferred from each well to a 96-well plate, and 100 μl of freshly prepared reaction mixture was added to each well. After 30 min of incubation at room temperature in the dark, the absorbance was determined at 490 nm using a Victor X3 multilabel reader. The amount of LDH was expressed as a percentage compared with the positive control treated with 2% Triton-X100.

Measurement of Intracellular Reactive Oxygen Species Generation

The intracellular accumulation of ROS was quantified using the fluorescent probe H2DCF-DA *in vitro*. The manufacturer's protocol was slightly modified in our current study. In brief, BEAS-2B cells were collected and a concentration of 2×10^5 cells/ml was achieved. Afterwards, the cells were labeled with 20 μM H2DCF-DA reagent and incubated for 30 min under normal cell culture conditions. After incubation, the cells were washed with 1× HBSS buffer, and then the cells were co-treated with PQ and *N*-acetyl-L-cysteine (NAC) in a 96-well black cell culture plate for 2 h. Intracellular fluorescence was detected using a Victor X3 multilabel reader with an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Western Blot Analysis

After co-treatment, cells were washed in cold 1× PBS and lysed on ice in RIPA lysis buffer (50 mM, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM Na_3Vo_4, and 1 mM NaF) containing proteases inhibitors (Santa Cruz Biotechnology Inc., CA, USA). Supernatants were collected by centrifugation at 10,000 rpm for 10 min at 4°C. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked using 5% dried skim milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then incubated overnight with primary antibodies against NRF2 (1:500) and β-actin (1:10,000) at 4°C. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz) and anti-mouse IgG antibodies were used as secondary antibodies for NRF2 and β-actin, respectively. To detect chemiluminescence signals, the ECL system (Supplier's information) was used, and images were captured using a ChemiDoc Imaging system (ChemiDoc XRS+ System with Image Lab Software; Bio-Rad).

qRT-PCR Analysis

BEAS-2B cells were treated with various concentrations of NG in the absence or presence of 0.2 mM PQ for the indicated time points. After treatment, total RNA was isolated using a RNA extraction kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using the Maxime RT PreMix kit (Intron Biotechnology, Korea) according to the manufacturer's protocol. Quantitative real-time-PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad) with iQTM SYBR Green Supermix (Bio-Rad) reagent and specific primer sets, which were purchased from Bioneer (PHS-001050, Accutarget human antioxidant Real-Time PCR primer set, Korea) [14, 26]. The antioxidant primer sets for catalase (*CAT*), glutathione reductase (*GSR*), peroxiredoxin (*PRDX*), glutathione S-transferase zeta (*GSTZ*), prostaglandin-endoperoxide synthase *1* (*PTGS1*), lactoperoxidase (*LPO*), and glutathione peroxidase (*GPX*) were used in this study. Other primer sets for *NRF2*, *HO-1,* and *NQO1* are listed in Table 1. The primers for *HO-1*, *NQO1,* and *GAPDH* genes were purchased from Bioneer (Daejeon, Korea), and an antioxidant primer set was also obtained from Bioneer (PHS-001050). QRT-PCRs were performed according to the manufacturer's protocol (Bio-Rad). In brief, samples were heated to 95°C for 5 min, followed by 40 cycles at 95°C (10 sec), 42°C (10 sec), and 72°C (20 sec). To ensure amplification of a single amplicon, the melting curve was assessed. The threshold cycle number (CT) of the gene was calculated, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. The delta-delta Ct values of the genes are represented as the relative fold induction. Data were compiled from three independent experiments.

Table 1. Primer sets used in the study.

Gene	Primer sequences	Amplicon sizes
HO-1 (F)	5′-GCAACCCGACAGCATGC-3′	245 bp
HO-1 (R)	5′-TGCGGTCGAGCTCTTCTG-3′	
NQO1 (F)	5′-CGCAGACCTTGTGATATTCCAG -3′	249 bp
NQO1 (R)	5′-CGTTTCTTCCATCCTTCCAGG -3′	
GAPDH (F)	5′-TCCCATCACCATCTTCCA-3′	380 bp
GAPDH (R)	5′-CATCACGCCACAGTTTCC-3′	

Small Interfering RNA (siRNA)

BEAS-2B cells were transiently transfected with control siRNA (sc-37007) or NRF2 siRNA (sc-37030) using a transfection reagent according to the manufacturer's protocol (Santa Cruz Biotechnology, CA, USA). In brief, cells were seeded in antibiotic-free normal growth medium and incubated at 37°C in a CO_2 incubator until the cells reached 60–80% confluency. For each transfection, 6 μl of siRNA duplex and siRNA transfection reagent (sc-29528) was diluted in 100 μl of siRNA tranfection medium (sc-36868). The siRNA duplex and siRNA transfection reagent were gently mixed by pipetting, and then incubated for 30 min at room temperature. In the meantime, cells were washed once with 2 ml of transfection medium. The mixture of siRNA duplex and siRNA transfection reagent was then added to the cells with 800 μl of transfection medium and then overlaid onto the cells. The cells were incubated at 37°C in a CO_2 incubator for 6 h. After incubation, the transfection medium was aspirated, and normal growth medium was added for an additional 24 h of incubation under normal cell culture conditions. Next, the transfected BEAS-2B cells were co-treated with PQ and NG for the indicated time periods. Finally, the cells were used for additional experiments such as western blot analysis.

Statistical Analysis

The statistically significant difference among three independent experiments was analyzed by a Student's *t*-test, assuming equal variance. The results were considered significant if the *P* value was <0.05. Data are expressed in graphs as the mean and SD.

Results

Naringenin Protects Against PQ-Induced Cell Death in BEAS-2B Cells

In order to investigate the cytoprotective effect of NG on PQ-exposed human bronchial epithelial BEAS-2B cells, we first tested the toxicity of NG itself (Fig. 1A). As shown in Fig. 1A, a variety of concentrations of NG from 5 to 100 μM did not cause any detrimental changes in viability of BEAS-2B cells (Fig. 1A). However, PQ treatment alone led to a dramatic reduction in cell viability by 30% and 70% at 48 and 72 h, respectively (Fig. 1B). Interestingly, co-treatment with 100 mM NG and PQ caused a recovery of the viability of BEAS-2B cells by 100% and 60% at 48 and 72 h, respectively. However, the changes in viability were not altered at 24 h by co-treatment with various concentrations of

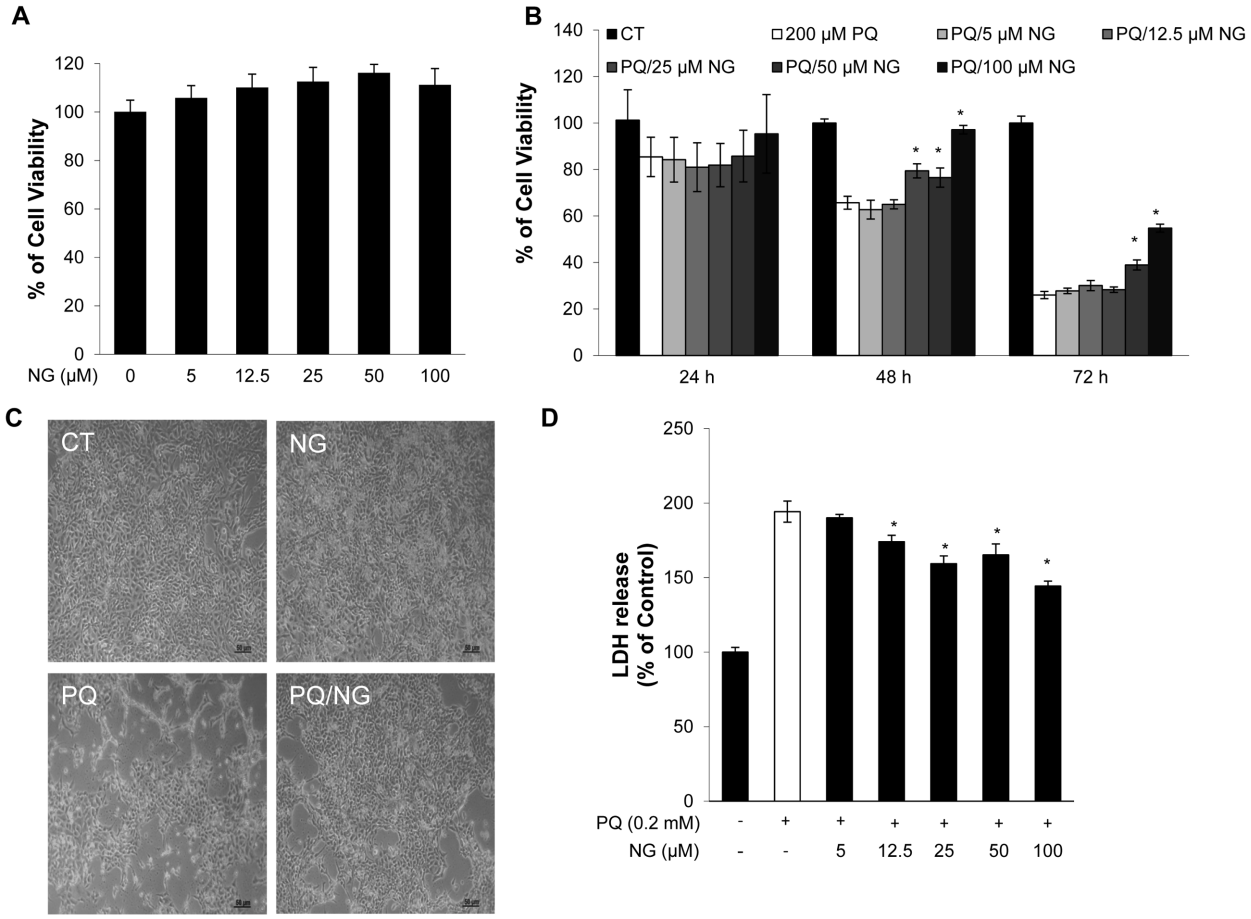

Fig. 1. Cytoprotective effect of NG on PQ-exposed BEAS-2B cells.
(**A**) In order to measure the toxicity of NG, BEAS-2B cells were treated with various concentrations of NG for 24 h. (**B**) To measure the viability of BEAS-2B cells, MTT analysis was performed. Cells were treated with either PQ only or PQ with various concentrations of NG for 24, 48, and 72 h. Asterisks (*) denote significant differences relative to 200 μM PQ-treated control, white bar ($p < 0.05$). (**C**) Morphological changes in PQ- and PQ/NG-treated BEAS-2B cells. The experiment was conducted by phase-contrast microscopy. The images shown here are representative of three independent experiments with similar results. (**D**) Reduction in PQ-induced LDH release by NG treatment in BEAS-2B cells. White bar indicates 200 μM PQ-treated control cells. All of the data were obtained from at least three independent experiments and presented as the means ± SD. Asterisks (*) indicate significant differences relative to PQ-treated control ($p < 0.05$).

NG (Fig. 2B). We also observed morphological changes in BEAS-2B cells that were treated either by PQ alone and/or co-treated with NG (Fig. 1C). As shown in Fig. 1C, control, untreated BEAS-2B cells were well attached to the bottom, even at 100% confluency; however, PQ-treated cells were shrunken and rounded, and approximately 40% of these cells were floating on the medium. However, after co-treatment with PQ and 100 μM NG under PQ-exposure conditions, BEAS-2B cells appeared to spread and were attached to the bottom. To assess the cellular membrane integrity after co-treatment with NG in PQ-treated BEAS-2B cells, we conducted a lactate dehydrogenase (LDH) assay (Fig. 1D). As shown in Fig. 1D, co-treatment with 100 μM NG and 0.2 mM PQ profoundly maintained the structure of the cell membrane.

Naringenin Diminishes the Intracellular ROS Generation

To investigate the role of NG in PQ-induced ROS generation, BEAS-2B cells were co-treated with PQ (0.2 mM) and different concentrations of NG (Fig. 2). A DCF-DA assay was used to measure the intracellular ROS generation. We observed that intracellular ROS generation was profoundly increased after PQ treatment relative to that in the control. Interestingly, a reduction in the intracellular ROS level was observed when the cells were co-treated with PQ and NG. To confirm the ROS scavenging effect of NG, we also

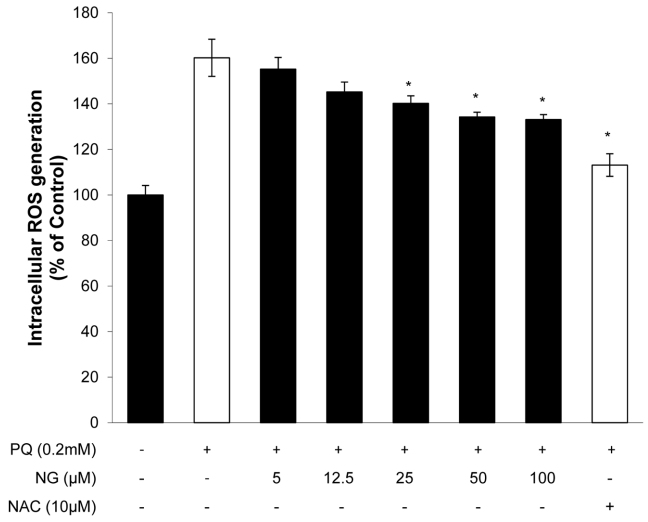

Fig. 2. Intracellular ROS scavenging effects of NG on PQ-induced BEAS-2B cells.

To investigate the ROS scavenging effect of NG, a DCF-DA assay was performed. The detailed experimental procedure is described in the Materials and Methods section. White bars indicate either 200 μM PQ-treated or PQ/NAC controls. All of the data were obtained from at least three independent experiments and presented as the means ± SD. Asterisks indicate statistically significant difference compared with PQ-treated control (*$p < 0.05$).

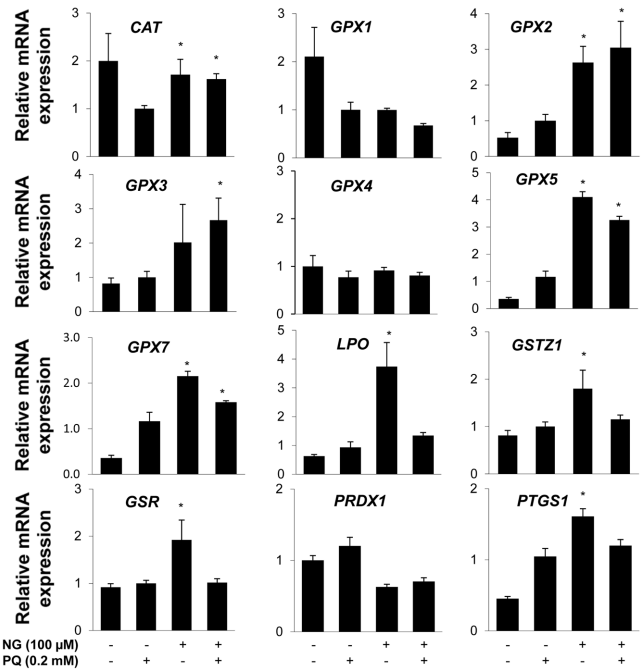

Fig. 3. Alteration in the expression of antioxidant-related genes by NG treatment in PQ-exposed BEAS-2B cells.

BEAS-2B cells were treated with NG in the presence of PQ for 6 h, total RNA was collected, and qRT-PCR was performed using specific primer sets. Expression value (fold change) was normalized by the housekeeping gene *GAPDH*. All of the data were obtained from at least three independent experiments and presented as the means ± SD. Asterisks indicate statistically significant difference compared with untreated controls. *$p < 0.05$.

treated the cells with *N*-acetyl-L-cysteine (NAC), which is a well-known ROS scavenger, as a positive control. These results suggest that NG acts as an efficient ROS scavenger under PQ-exposed conditions.

Naringenin Induces the Expression of Antioxidant-Related Genes in PQ-Exposed BEAS-2B Cells

To further determine whether NG is involved in the expression of antioxidant-related genes, we measured the expression of antioxidant-related genes by qRT-PCR. Interestingly, the expression of several antioxidant-related genes, including *CAT*, *GPX2*, *GPX5*, *GPX7*, *LPO*, *GSTZ1*, *GSR*, and *PTGS1*, was induced by NG treatment itself; whereas the expression of *GPX1*, *GPX3*, *GPX4*, and *PRDX1* was not significantly altered (Fig. 3). Under PQ-exposed conditions, the expression of *CAT*, *GPX2*, *GPX3*, *GPX5*, and *GPX7* mRNAs was profoundly induced compared with that in the only PQ-treated cells. Next, we assessed the expression of NRF2 and its downstream target genes. As shown in Fig. 4A, the expression of *NRF2* and its target genes *HO-1* and *NQO1* was induced by NG co-treatment or NG treatment itself. In order to check the nuclear translocation of NRF2, we collected the cytosolic and nuclear protein separately, and NRF2 protein expression was quantified by western blot analysis (Fig. 4B). A prominent NRF2 expression in the nucleus was detected in BEAS-2B cells treated with both PQ (0.2 mM) and NG (100 μM), whereas NRF2 expression was markedly lower in the cytoplasm (Fig. 4B). The mRNA expression of *HO-1* and *NQO1* was remarkably induced by NG co-treatment in PQ-treated cells as well as by NG treatment itself (Figs. 4C and 4D). To further assess the mediating role of NRF2 induction after NG co-treatment in PQ-exposed cells, knockdown of the NRF2 expression by NRF2 siRNA transfection in BEAS-2B cells was performed (Fig. 5A). In scrambled control siRNA transfected cells, *HO-1*, *NQO1*, and *NRF2* expression was greatly induced by NG treatment itself and by co-treatment of NG with PQ. However, the expression of *NRF2* and its target genes *HO-1* and *NQO1* was drastically reduced in NRF2 siRNA-transfected BEAS-2B cells. The results suggested that NRF2 activation is an essential event in the NG-mediated protection against ROS insult. In addition, we tested the viability of PQ-exposed

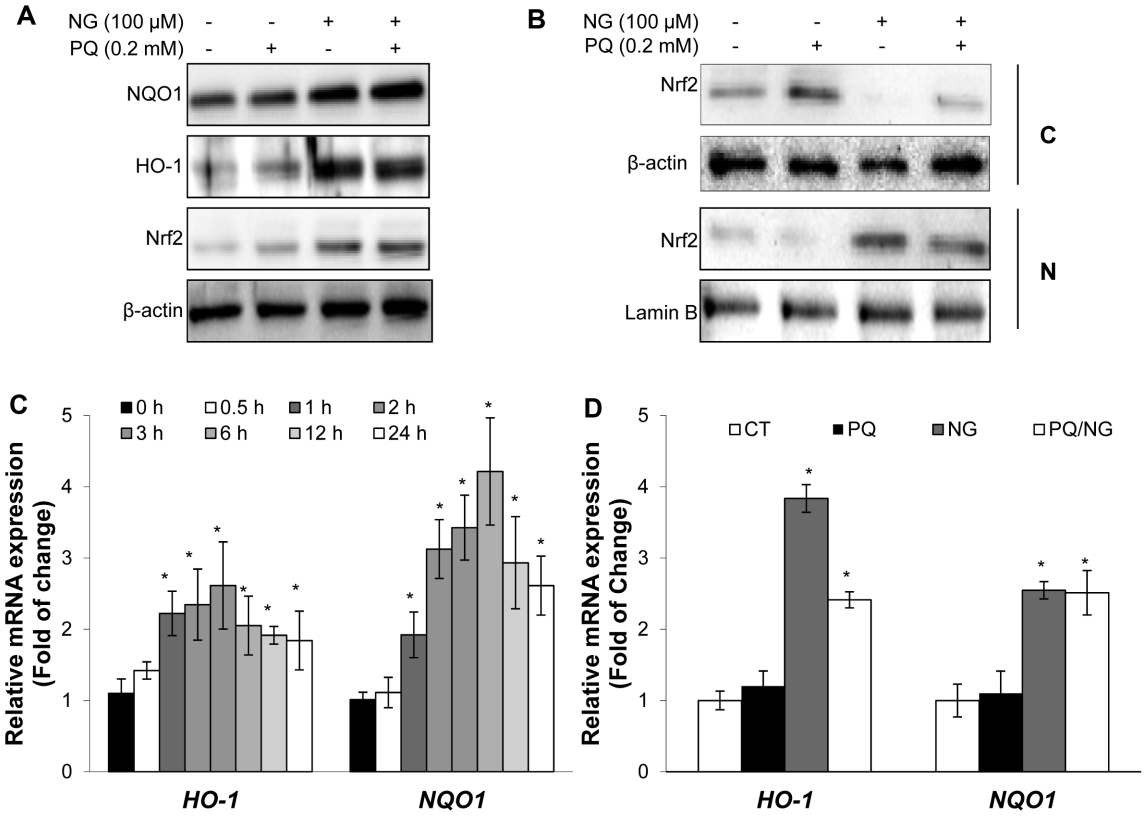

Fig. 4. Activation of *NRF2* and its downstream target genes *HO-1* and *NQO1* by NG treatment in PQ-exposed BEAS-2B cells.
(**A**) Protein expression of NRF2, HO-1, and NQO1 in BEAS-2B cells treated with 0.2 mM PQ and 100 μM NG for 6 h. (B) In order to test NRF2 nuclear translocation, PQ- or PQ/NG-treated cells were fractionated and western blots were performed. C and N indicate the cytosolic and nuclear fractions, respectively. (**C**) BEAS-2B cells were treated with 100 μM NG for the indicated times and mRNA expressions were quantified. Asterisks denote statistically significant difference compared with untreated control cells (*$p < 0.05$). (**D**) Cells were treated with different concentrations of NG with 0.2 mM PQ. Six hours later, total RNA was collected and qRT-PCR was performed using *HO-1* and *NQO1* primer sets. The expression value was normalized by the housekeeping gene *GAPDH*. All of the data were obtained from at least three independent experiments and presented as the means ± SD. Asterisks indicate statistically significant difference compared with untreated control cells (*$p < 0.05$).

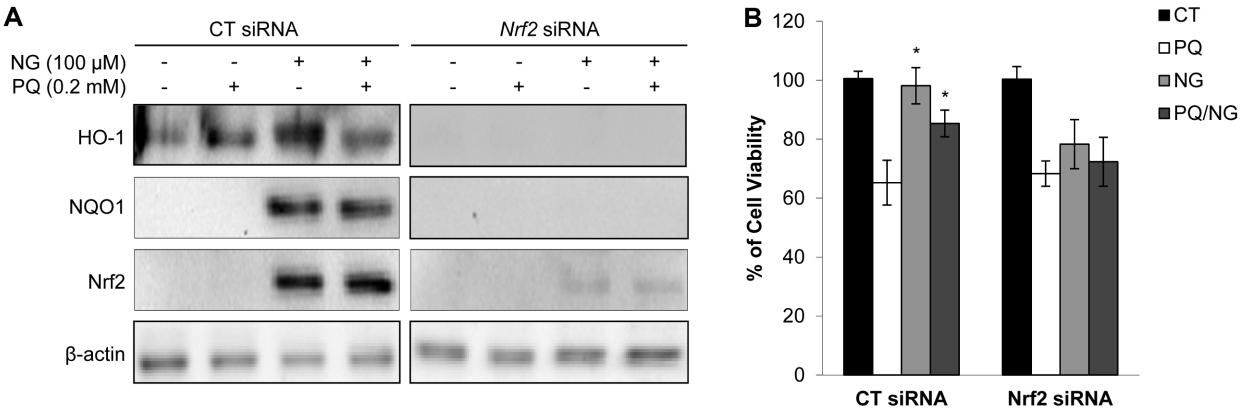

Fig. 5. Pivotal role of NRF2 in NG-mediated cytoprotection against PQ-induced loss of viability in BEAS-2B cells.
(**A**) NRF2 siRNA suppresses NG-induced NRF2 induction and its target genes *HO-1* and *NQO1* under PQ-exposed conditions. (**B**) Cell viability was measured after knockdown of NRF2 in cells with PQ-mediated cytotoxicity. All of the data were obtained from at least three independent experiments and presented as the means ± SD. Asterisks indicate statistically significant difference compared with PQ-treated controls (*$p < 0.05$).

BEAS-2B cells under NRF2 knockdown conditions using small interfering RNA. As shown in Fig. 5B, NG treatment alone and co-treatment of NG with PQ can significantly rescue the viability of BEAS-2B cells under control siRNA conditions. However, NG could not prevent cell death in NRF2 knocked down cells treated with PQ.

The combined data suggested that the phytochemical NG has a cytoprotective effect against PQ-induced toxicity in human bronchial epithelial BEAS-2B cells *via* modulation of NRF2-mediated antioxidant gene regulation.

Discussion

Scientists have demonstrated that the nuclear factor erythroid-2 related factor 2 plays a pivotal role in cell survival through the antioxidant signaling pathway [11, 12, 23]. Numerous natural products and synthetic compounds have been considered and identified as activators of the NRF2-ARE (antioxidant response element) pathway in oxidative-related human diseases such as cancer, diabetes, cardiovascular dysfunction, and fibrotic diseases. In order to discover potent candidates against PQ-induced ROS stress, we screened various phytochemicals. NG is one of the potential candidates, and it is derived from various fruits such as cherries. Potent activities of NG, such as its anti-inflammatory, antioxidant, and antitumor properties, have been reported in various human diseases [18, 22].

It is known that flavonoids can chelate Fe and Cu, which are derived primarily from the presence of 3-hydroxypyran-4-one and secondarily from the presence of 5-hydroxy and 4-carbonyl groups in the C ring, rather than from the 3′,4′-dihydroxy group in the B ring. NG has 5-hydroxy and 4-carbonyl groups in the C ring that may interact with Cu and Fe ions [21]. In this study, we demonstrated that NG can protect against ROS-mediated cell toxicity through inhibition of intracellular ROS generation and intracellular ROS scavenging. We can speculate that ROS scavenging by NG may be through its 5-hydroxy and 4-carbonyl groups in the C ring, which may interact with Cu and Fe ions. Therefore, NG plays a role in cellular protection under stress conditions partly by its radical-scavenging and iron-chelating properties.

The potential role of NG has been reported in the past few years. It has been reported that NG can induce NRF2 expression under various stress conditions [6, 8]. In this study, we found that NG can induce NRF2 expression and promote its nuclear translocation. In addition, NG can up-regulate the expression of NRF2 downstream target genes such as *HO-1* and *NQO1* under PQ-induced ROS stress conditions. A well-established mechanism of NRF2 activation is dependent on the external signals such as xenobiotics, antioxidants, metals, and UV irradiation [17]. Activated and stabilized NRF2 translocates to the nucleus, binds to the ARE sites on the promoter regions of the target genes, and regulates ARE-mediated antioxidant-related gene expression. Kelch-like ECH-associated protein 1 (Keap1) binds the amino terminus region of NRF2 and negatively regulates NRF2 activation [17]. Another possibility of activation of NRF2 by NG is *via* the dissociation of the NRF2-Keap1 inhibitory complex that allows the NRF2 protein to accumulate in the nucleus. However, the probable event was not investigated in the present study because the aim of this study was not to identify this mechanism; however, the classical phenomenon should be elucidated in detail. We further tested whether the activation of NRF2 also induces antioxidant gene expression in BEAS-2B cells. NRF2 small interfering RNA hindered NRF2 translocation, and cells were co-treated with PQ and NG, or NG alone. Contrary to the control siRNA-treated cells, NRF2 siRNA successfully blocked NRF2 protein expression and its target gene expression. Under *NRF2* gene knockdown conditions, NG could not recover the viability of BEAS-2B cells that were exposed to PQ. Thus, NRF2 plays an important role in cytoprotection induced by NG treatment against PQ-induced toxicity in BEAS-2B cells.

Interestingly, NG can also effectively induce the expression of many antioxidant-related genes, such as *CAT*, *GPX2*, *GPX3*, *GPX5*, and *GPX7*, but not the expression of *GPX1*, *GPX4*, *LPO*, *GSTZ1*, *GSR*, *PRDX1*, and *PTGS1*, during PQ-induced ROS stress in BEAS-2B cells. Our previous data have reported that NRF2 plays a pivotal role in the activation of antioxidant-related genes and in the protection against PQ-induced cellular toxicity [14, 19, 26]. Zerin *et al*. [26] demonstrated that PQ treatment can induce the expression of various antioxidant-related genes including the glutathione peroxidase gene family [14, 19]. After treatment with the antioxidant quercetin under PQ-treated conditions, among all of the GPX family members, only the expression of *GPX5* was significantly increased after treatment with both PQ and quercetin [26]. In the present study, the expression of *GPX5* was upregulated 4-fold and 3-fold by NG treatment only and PQ/NG co-treatment, respectively. The *in vivo* study also demonstrated that GPX5 is a potent ROS scavenger and protects the mouse epididymis from oxidative stress [3]. Previous studies have also demonstrated that NG has a protective effect against cytotoxicity induced by various chemicals *via* the activation of antioxidant-related genes [20]. Therefore, we can

speculate that *GPX5* is a key antioxidant gene among the GPX family members under PQ-induced ROS conditions. This speculation also indicates that NG may also activate the intracellular signaling pathways, either the NRF2/ARE pathway or the NRF2-independent pathway, to induce activation of the cellular defense-related genes [9].

In summary, the present study demonstrates the cellular protective mechanism of NG against PQ-induced cytotoxicity in human bronchial epithelial BEAS-2B cells through NRF2 activation, following activation of *HO-1*, *NQO-1*, and other antioxidant genes. These results provide the first evidence that NG inhibits PQ-induced ROS accumulation and exerts cellular protection *via* activation of the NRF2-ARE mechanism in PQ-exposed BEAS-2B cells.

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