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

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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% confluence 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-propanenitrile) 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 × 10^4 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 1x PBS and lysed on ice in RIPA lysis buffer (50 mM, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl), 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 from Bioneer (Daejeon, Korea), and an antioxidant primer set was also obtained from Bioneer (PFS-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.

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 CO₂ 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 transfection 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 CO₂ 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 PQ was recovered 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 PQ.
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...
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 \( \text{CAT} \), \( \text{GPX2} \), \( \text{GPX5} \), \( \text{GPX7} \), \( \text{LPO} \), \( \text{GSTZ1} \), \( \text{GSR} \), and \( \text{PTGS1} \), was induced by NG treatment itself; whereas the expression of \( \text{GPX1} \), \( \text{GPX3} \), \( \text{GPX4} \), and \( \text{PRDX1} \) was not significantly altered (Fig. 3). Under PQ-exposed conditions, the expression of \( \text{CAT} \), \( \text{GPX2} \), \( \text{GPX3} \), \( \text{GPX5} \), and \( \text{GPX7} \) 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 \( \text{HO-1} \) and \( \text{NQO1} \) was 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, \( \text{HO-1} \), \( \text{NQO1} \), 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 \( \text{HO-1} \) and \( \text{NQO1} \) 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 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.

**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 \( \text{GAPDH} \). 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. \(^* p < 0.05\).
**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).
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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.

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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References

