

Oligomeric proanthocyanidins protects A549 cells against H₂O₂-induced oxidative stress via the Nrf2-ARE pathway

CHAO SUN, WEIGUO JIN and HONGCAN SHI

Department of Cardiothoracic Surgery, Northern Jiangsu People's Hospital
Affiliated to Yangzhou University, Yangzhou, Jiangsu 225001, P.R. China

Received February 6, 2016; Accepted April 7, 2017

DOI: 10.3892/ijmm.2017.2971

Abstract. Oxidative signaling and oxidative stress contribute to aging, cancer and diseases resulting from lung fibrosis. In this study, we explored the anti-oxidative potential of oligomeric proanthocyanidins (OPCs), natural flavonoid compounds. We examined the protective effects of OPCs against hydrogen peroxide (H₂O₂)-induced oxidative stress in non-small cell lung cancer cells (A549). We demonstrated that OPC markedly attenuated H₂O₂-induced A549 cell viability, as shown by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. At the same time, OPC inhibited H₂O₂-induced oxidative stress by significantly increasing the activities of superoxide dismutase, catalase and glutathione, and reducing the levels of reactive oxygen species (ROS) and malondialdehyde (MDA). Treatment of the A549 cells with OPC significantly promoted the nuclear translocation of NF-E2-related factor 2 (Nrf2) and significantly enhanced the expression of its target genes [heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1) and thioredoxin reductase 1 (TXNRD1)] with different fold change values at both the mRNA and protein level. The knockout of Nrf2 using CRISPR/Cas9 technology attenuated OPC-mediated ARE gene transcription, and almost abolished the OPC-mediated protective effects against H₂O₂-induced oxidative stress. On the whole, our study suggests that OPC plays an important role in controlling the antioxidant response of A549 cells via the Nrf2-ARE pathway.

Introduction

Oligomeric proanthocyanidins (OPCs) are extensively distributed in the plant kingdom and are present in high

concentrations in certain plant-based foods and beverages (1). Previous studies have suggested that OPCs provide health benefits, such as a reduced risk of vascular disease and certain types of cancer (2,3). OPCs have also been shown to alleviate EtoH-induced liver steatosis and injury in mice, while increasing superoxide dismutase (SOD) activity (4). OPCs extracted from apples have also been shown to exert anti-oxidant effects (5). However, the mechanisms through which OPCs protect cells against oxidative stress require clarification. All living organisms are constantly challenged by internal and external oxidative stress, such as those from drugs, xenobiotics, heavy metals and ionizing radiation, resulting in the increased production of reactive oxygen species (ROS) (6). ROS include superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and the extremely reactive hydroxyl radical (OH[•]), with each species displaying a specific range of action and distinct molecular targets (7,8). ROS exert significant effects in cancer cells, such as stimulating cellular growth, promoting mutations and inducing resistance to anticancer agents (9). However, a previous study reviewed the possible therapeutic effects of ROS, suggesting that increased intrinsic ROS-induced stress may prove beneficial as it may enhance the killing effect of therapeutic drugs, as continuous ROS insults decrease the tolerance of cancer cells (10). Others have reported however, that ROS derived from circulating inflammatory cells contribute to the induction of lung injury (11).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is commonly known to play a role in the transcriptional regulation of genes encoding antioxidant proteins under stress conditions (12,13). Target genes of Nrf2 are involved in the glutathione synthesis, the elimination of ROS and xenobiotic metabolism (14,15) and some of these enzymes include SOD, glutathione peroxidase (GSH-Px), glutathione reductase, catalase (CAT), heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1) and thioredoxin reductase 1 (TXNRD1). Under normal conditions, Nrf2 is constantly polyubiquitinated by the Keap1-Cul3 E3 ligase and degraded by the 26S proteasome (16). In the presence of electrophiles or ROS, the ability of the Keap1-Cul3 E3 ligase to target Nrf2 for degradation becomes impaired, stabilized Nrf2 accumulates in the nuclei, heterodimerizes with small Maf proteins and activates target genes for cytoprotection through the antioxidant response element (ARE) with a consensus sequence 5'-TGACNNNGC-3' (17,18). The increased expression of Nrf2

Correspondence to: Professor Hongcan Shi, Department of Cardiothoracic Surgery, Northern Jiangsu People's Hospital Affiliated to Yangzhou University, 98 West Nantong Road, Yangzhou, Jiangsu 225001, P.R. China
E-mail: hongcanshi01@163.com

Key words: oxidative stress, oligomeric proanthocyanidin, hydrogen peroxide, NF-E2-related factor 2, CPISPR/Cas9

target genes and the increased stability of Nrf2 caused by somatic mutations in Nrf2 and Keap1 are well documented in human cancer (19). These data indicate that the Nrf2-ARE pathway plays an important role against oxidative stress in cancer cells.

In this study, we investigated the anti-oxidative potential of OPCs against H₂O₂-induced oxidative stress in A549 non-small cell lung cancer cells. OPCs prevent oxidative stress by the accumulation of Nrf2 protein in A549 cells. Further research will be crucial in determining how this inhibitor mediates lung protection and its use as a clinical approach in lung disease.

Materials and methods

Chemicals and reagents. OPC (purity $\geq 98\%$) was purchased from Zelang Biological Technology Co., Ltd. (Nanjing, China). H₂O₂ was obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco Industries, Inc. (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin, L-glutamate, MK-801 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. The DCFH-DA ROS assay kit was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). GSH-Px, CAT and SOD assay kits were procured from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The bicinchoninic acid (BCA) protein reagent was from the Beyotime Institute of Biotechnology Co. Ltd., Shanghai, China).

Cell culture and treatment. For this study, A549 cells were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (Wako, Osaka, Japan). The cells were cultured in a humidified incubator containing 5% CO₂ and 95% air at 37°C. The cells were divided into 3 groups based on the treatments as follows: the control group (cells treated with culture medium), H₂O₂ group (cells exposed to H₂O₂ for 12 h at a final concentration of 200 μ M) and the H₂O₂ + group (cells pre-treated with 50 mg/l OPC for 5 h and then exposed to 200 μ M H₂O₂ for 12 h). The cells were subjected to the different treatments and were then subjected to serial analyses, including flow cytometry (FCM), MTT, RT-qPCR and western blot analysis.

Assessment of the viable number of cells. Cell viability was performed using the MTT colorimetric assay. Following cell treatment, the medium was removed, and the cells were incubated with 20 μ l of 5 mg/ml MTT solution for 4 h at 37°C. The dark blue formazan was dissolved with 150 μ l of DMSO, and the absorbance was measured at 570 nm using a microplate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was expressed as a percentage of the untreated controls.

Western blot analysis. The cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with a protease and phosphatase inhibitor cocktail on ice for 15 min,

sonicated and centrifuged at 13,000 x g for 15 min. The protein content of the supernatants was measured by BCA reagent (Pierce, Rockford, IL, USA). For the examination of Nrf2 expression, nuclear proteins and cytoplasmic proteins were separated using a Bioepitope Nuclear and Cytoplasmic Extraction kit (Bioworld Technology, St. Louis Park, MN, USA) according to the manufacturer's instructions. Cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were probed with the following primary antibodies (Abs): anti-TXNRD1 (ab16847; 1:1,000; Abcam, Cambridge, MA, USA); anti-Nrf2 (#12721; 1:1,000; Cell Signaling Technology, Danvers, MA, USA); anti-tubulin (ab7291; 1:5,000); anti-HO-1 (ab13248; 1:1,000); lamin B (ab8980; 1:1,000) and anti-NQO1 (ab34173; 1:1,000) (all from Abcam, Cambridge, MA, USA). This was followed by detection using HRP-conjugated secondary antibodies to rabbit IgG (GE Healthcare, Piscataway, NJ, USA).

RT-qPCR. Total RNA was extracted from the A549 cells using TRIzol reagent and then reverse transcribed into cDNA using the QuantScript reverse transcription kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Amplification was conducted using a SYBR-Green I PCR kit (Roche, Indianapolis, IN, USA). The PCR reaction conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 amplification cycles of 95°C for 10 sec, 55°C for 10 sec, 72°C for 15 sec, and a final extension at 72°C for 10 min. β -actin was used as an internal reference gene. Relative values for mRNA levels were calculated using the following formula: fold change = $2^{-\Delta\Delta C_q}$. The primers used for each gene are listed in Table I.

Intracellular ROS staining. The cells were incubated with 300 nM dichloro-dihydro-fluorescein diacetate (DCF-DA) (Sigma-Aldrich) for 10 min at 37°C. Following 2 washes in phosphate-buffered saline (PBS)-1X, DCF-DA fluorescence was analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software.

Measurement of intracellular GSH-Px, CAT and SOD levels. The A549 cells were plated in 6-well plates at a density of 10×10^4 cells/ml in 2.5 ml of culture medium. After the cells were treated in accordance with the experimental design, the cells were then harvested, disrupted ultrasonically on ice and centrifuged at 2,500 x g for 10 min at 4°C. The supernatants were collected and stored -20°C for subsequent analysis. The levels of SOD, GSH-Px and CAT were detected using a Wallac 1420 microplate spectrophotometer (Perkin Elmer, Waltham, MA, USA) using commercially available assay kits (Jiancheng Bioengineering Institute) following the manufacturers' instructions.

Determination of the activity of malondialdehyde (MDA). A reaction mixture containing 0.2 ml cell lysate and 4.2 ml reaction buffer was treated by ultrasound for a certain time using a 22 kHz ultrasound generator (HN-1000Y; Shanghai Hanno Instrument Corp., Shanghai, China) equipped with a tapered horn tip (10-mm end diameter) and boiled for 10 min in water bath, then cooled down and centrifuged at 4,000 rpm for

Table I. Sequence of the primers used in RT-qPCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Nrf2	GAGACAGGTGAATTTCTCCCAAT	GGGAATGTGGGCAACCTGGG
HO-1	CAGGCAGAGAATGCTGATTC	GCTCTTCTGGGAAGTAGACAGG
NQO1	AAGAAAGGATGGGAGGTGGT	GCTTCTTTTGTTCAGCCACA
TXNRD1	GGAAGTAGATGGGGTCTCGG	TCTTGCAGGGCTTGTCTCTAA
β-actin	GATCATTGCTCCTCTGAGC	ACTCCGCTTGCTGATCCAC

Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD1, thioredoxin reductase 1.

10 min. the supernatants were measured using a kit purchased from the Nanjing Jiancheng Bioengineering Institute according to the thiobarbituric acid method, which is based on the fact that MDA reacts with thiobarbituric acid to form thiobarbituric acid reactive substances (TBARS) with a maximum absorbance at 530 nm. The experiments were performed in triplicate.

Knockout of the Nrf2 gene using the CRISPR/Cas9 mediated genome. Target sequences for CRISPR interference (20) were designed at CRISPR direct (<http://crispr.dbcls.jp/>). The target sequences for human Nrf2 were TAGTTCATGAGCGTGA TGAT (exon 3) and TGCCATAATTGTTACACATT (exon 4). Two oligonucleotides with *Bbs*I restriction sites for guide RNAs (gRNAs) were synthesized by Sheng Gong (Shanghai, China) and cloned into the PX330 CRISPR/Cas9 vector (Addgene, Cambridge, MA, USA). The cells were transfected the two PX330 guides and PMT-puro by using PolyJet reagent (Invitrogen, Carlsbad, CA, USA). The cells were selected with 1.5 µg/ml puromycin and stable clones were maintained in 0.5 µg/ml puromycin. The allele-specific primers were designed as follows: forward, GATTCTTGTGAAGCAG TCCAGC and reverse, ATCCAGTGACTTCTTGAATGCTT. Lastly, the potential positive clones from PCR results were selected and this was followed by electrophoresis on a 1% agarose gel. The positive clones will loss about 1000 bp DNA fragment.

DNA gel electrophoresis. First, the cells were spinned down in the PCR plate, and the supernatant was discard, leaving 10 µl medium. The cells were then lysed using single cell lysis buffer (1 mM EDTA, 10% Tween-20) and then boiled at 65 °C for 2 h; 95 °C for 30 min. PCR was then performed with cell lysis buffer and allele-specific primers followed by electrophoresis on a 1% agarose gel. The DNA gel was scanned using a gel imaging system (Tannon 3500R; Tanon Science & Technology Co., Ltd., Shanghai, China).

Luciferase assay. The NQO1-ARE promoter was cloned into pGL3-Basic (Promega, Madison, WI, USA) vector by standard methods. For the luciferase assay, the A549 cells were transiently transfected with the luciferase construct and β-galactosidase (as an internal control). The cells were transfected using PolyJet reagent (Invitrogen) according to the manufacturer's instructions. Luciferase activity was determined using a luminometer. The medium was removed from the cells, and the cells were then lysed in 100 µl of extrac-

tion buffer (100 mM KPO₄, pH 7.4, 4.0 mM ATP, 1.5 mM MgSO₄, 1.0 mM dithiothreitol and 0.1% Triton X-100). Cell lysates (25 µl) were added to 350 µl of luciferase assay buffer (100 mM KPO₄, pH 7.4, 4.0 mM ATP, and 1.5 mM MgSO₄) and then 100 µl of 1.0 mM D-Luciferin was injected into each culture tube using a luminometer that integrated the luminescence over 10 sec. β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich) as a substrate. Briefly, the cell lysates were incubated with ONPG (0.4 mg/ml) in reaction buffer (0.1 M sodium phosphate pH 7.5, 10 mM KCl, 1 mM MgCl₂) for 1 h and the absorbance was then measured at 405 nm. The data are expressed as a ratio of luciferase to β-galactosidase activity.

Statistical analysis. All data are presented as the means ± standard deviation (SD). Statistical analysis was carried out using one-way ANOVA followed by the Scheffe test using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was set at *p* < 0.05.

Results

OPC protects against cell death and attenuates oxidative stress in A549 cells exposed to H₂O₂. We first examined the potential effects of OPC in A549 cells. The results of MTT assay for cell survival shown in Fig. 1A demonstrated that the viability of the A549 cells was not significantly decreased when the cells were treated with OPC at the concentration of ≤50 mg/l. However, at the concentration of ≥100 mg/l OPC, A549 cell viability was slightly, but significantly decreased (Fig. 1A). Subsequently, H₂O₂-induced oxidative damage was investigated in the A549 cells by MTT assay. The viability of the A549 cells decreased following exposure to H₂O₂ in a dose-dependent manner. Following 12 h of exposure to 100 µM H₂O₂, cell viability decreased to 70% of the control, whereas 200 µM decreased cell viability to 50% and this concentration was utilized for further experiments (Fig. 1B). Significantly, treatment with OPC at 1-50 mg/l (non-cytotoxic concentrations) markedly attenuated the H₂O₂-induced decrease in A549 cell viability (Fig. 1C). We then examined the effects of OPC on the activities of a series of phase II metabolizing enzymes and antioxidants. The results demonstrated that the enzyme activities of SOD, CAT and GSH-Px were significantly decreased in the H₂O₂-treated A549 cells, and that OPC at 50 mg/l enhanced their activities (Fig. 1D-F). We also determined the contents of ROS and MDA within the A549 cells. We found that the

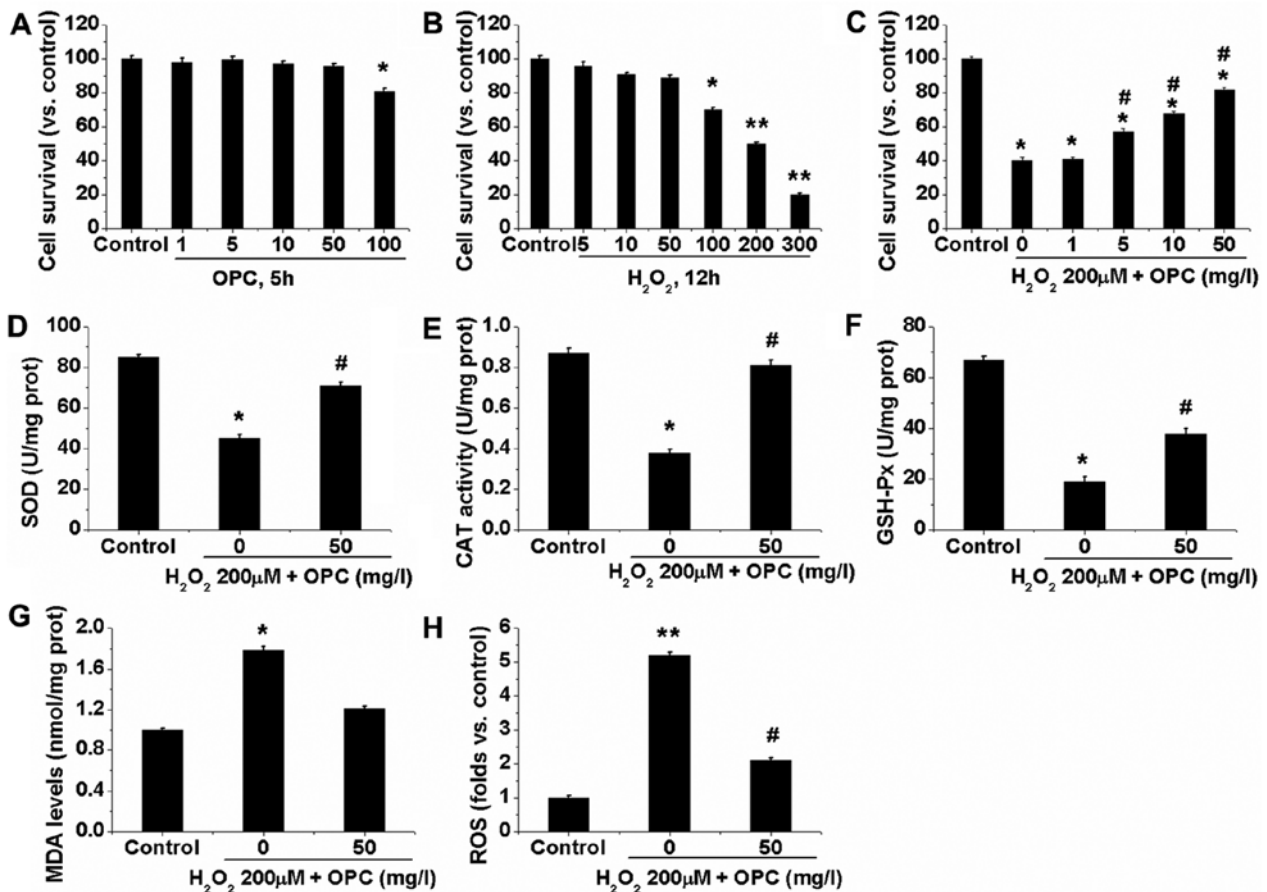


Figure 1. Oligomeric proanthocyanidins (OPCs) alleviate H₂O₂-induced oxidative stress in A549 cells. A549 cells (A and C) were treated with the indicated concentrations of OPC for 5 h and/or H₂O₂ for 12 h, and cell survival was examined by MTT assay. (B) Cells were exposed to various concentrations of H₂O₂ and cell viability was examined. Experiments were repeated 3 times with similar results obtained (same for all figures). For each assay, n=6 (same for all figures). Bars indicate the means \pm SD (same for all figures). Effects of OPC on superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in H₂O₂-exposed A549 cells. Cells were pre-treated with 50 mg/l OPC for 5 h and then exposed to 200 μ M of H₂O₂ for 12 h (D-G). (H) Effect of OPC on H₂O₂-induced ROS production in A549 cells. DCFH-DA fluorescence reflects the level of ROS. The values are expressed as the means \pm SD (n=3). *p<0.05 vs. control group; **p<0.01 vs. control group; #p<0.05 vs. H₂O₂ only group.

levels of ROS and MDA were significantly elevated in the H₂O₂-exposed cells. Similarly, OPC at 50 mg/l significantly decreased the contents of ROS and MDA (Fig. 1G and H). These results indicated that OPC exerted potent antioxidant effects on H₂O₂-exposed A549 cells.

OPC increases the transcriptional activity of Nrf2 and the Nrf2-dependent antioxidant response of A549 cells exposed to H₂O₂. In order to elucidate the potential mechanisms responsible for the protective effects of OPC against H₂O₂-induced oxidative stress, the A549 cells were exposed to H₂O₂ for 12 h following treatment or not with OPC. We then examined the mRNA and protein expression of Nrf2 and Nrf2 target genes. We found that H₂O₂ increased the mRNA expression of Nrf2 and Nrf2 target genes and this increase was further enhanced by OPC (Fig. 2A). Furthermore, the results of western blot analyses revealed that the nuclear protein expression of Nrf2 was significantly increased by H₂O₂ and OPC; however, the cytoplasmic protein expression of Nrf2 was not altered by H₂O₂ and OPC (Fig. 2B). As regards HO-1, NQO1 and TXNRD1, their protein expression was significantly increased by OPC (Fig. 2C). Subsequently, to investigate the effects of OPC on Nrf2 transcriptional activity under conditions of H₂O₂-

induced oxidative stress, the enzymatic activity of NQO1 was also examined. As observed with the mRNA expression of NQO1, which increased following treatment with OPC in the A549 cells, the enzymatic activity of NQO1 was also increased in the A549 cells following treatment with OPC (Fig. 2D). Taken together, these results indicate that OPC promotes the nuclear translocation of Nrf2, leading to the enhanced expression of its target genes implicated in the protective effects of OPC against H₂O₂-induced oxidative stress in A549 cells.

Effects of the knockout of Nrf2 in A549 using the CRISPR/Cas9 system. As Nrf2 was above shown to participate in the protective effects of OPC against oxidative stress, we knocked out Nrf2 in the A549 cells in order to further explore the overall influence of the process. We used the CRISPR/Cas9 system that has been reported to efficiently disrupt genes in cells (21). As a result, we obtained 2 Nrf2 knockout A549 clones, 4# and 5#. The DNA sequences of the 2 clones revealed a deletion of the 1173 and 1162 bases in both alleles (Fig. 3A and B). DNA gel-image indicated that Nrf2 deletion >1,000 bases between exon 3 and exon 4 (Fig. 3C). Western blot analysis revealed that Nrf2 was completely knocked out in the 2 clone cells by the absence of Nrf2 expression (Fig. 3D).

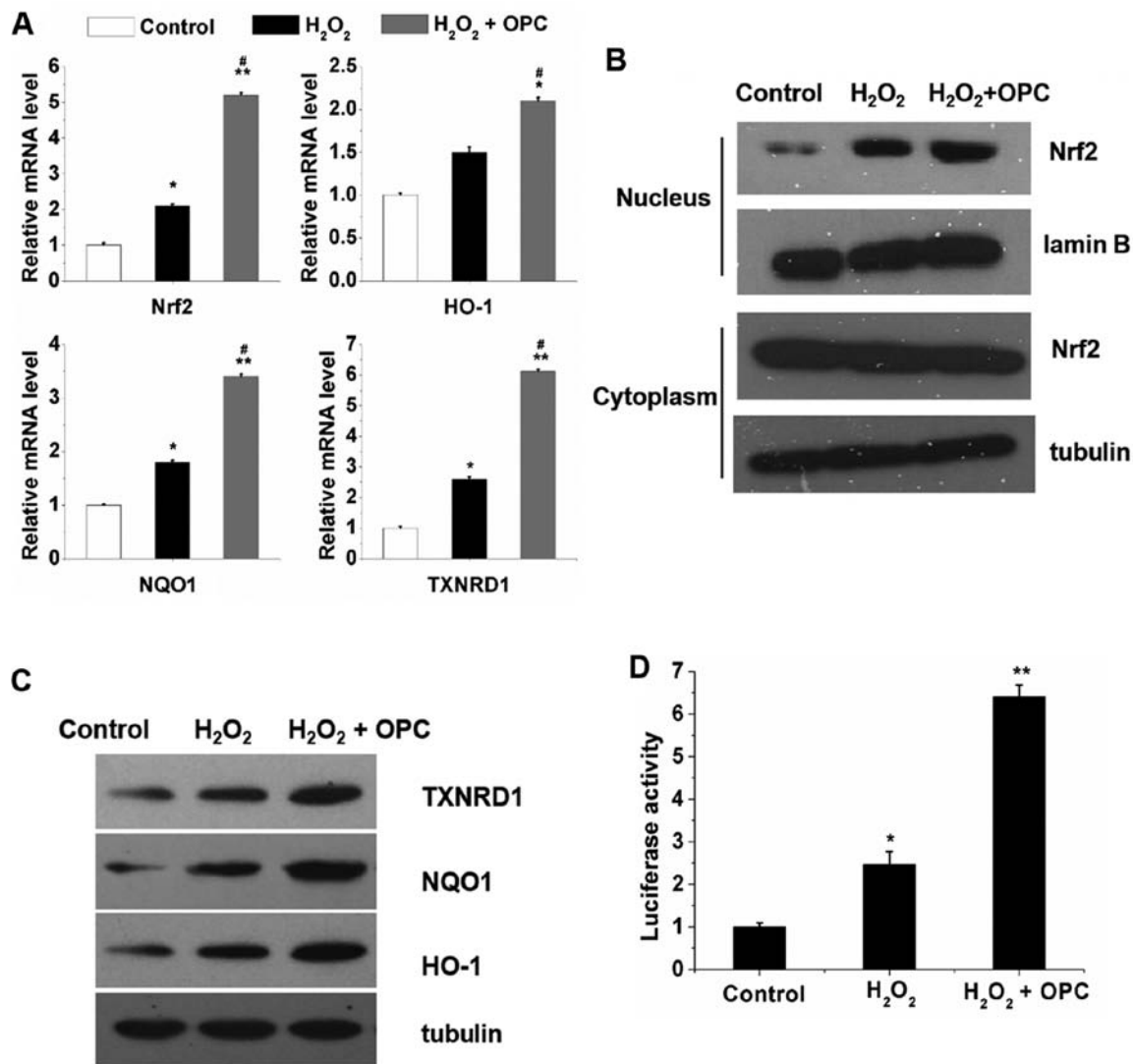


Figure 2. Oligomeric proanthocyanidins (OPCs) increase the transcriptional activity of NF-E2-related factor 2 (Nrf2) and the Nrf2-dependent antioxidant response. A549 cells were pre-treated with 50 mg/l OPC for 5 h and then exposed to 200 μ M H₂O₂ for 12 h. RNA was extracted and RT-qPCR was performed. Values were normalized to actin, and the controls were set equal to 1. *p<0.05 vs. control group; #p<0.05 vs. H₂O₂ only group. A549 cells were exposed to 200 μ M H₂O₂ for 12 h with or without 50 mg/l OPC. Western blot analyses for protein abundance of Nrf2 in the cytoplasm and nucleus. A549 cells were exposed to 200 μ M H₂O₂ for 12 h with or without 50 mg/l OPC. Cell lysates were subjected to western blot analysis using Nrf2, NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase-1 (HO-1) and thioredoxin reductase 1 (TXNRD1) antibodies. A549 cells were transfected with expression plasmids for NQO1-ARE promoter-firefly luciferase and β -galactosidase as an internal control for 36 h then exposed to H₂O₂ (200 μ M) for 12 h, with or without pre-treatment with OPC (50 mg/l). Both firefly and β -galactosidase were measured, and results are presented as firefly/ β -galactosidase activity. *p<0.05 vs. control group; **p<0.01 vs. control group; #p<0.05 vs. H₂O₂ only group.

Nrf2 mediates H₂O₂-induced antioxidant activity. We then characterized the function of OPC in A549 cells in which Nrf2 had been knocked out. Note that in the A549 cells in which Nrf2 was knocked out, H₂O₂-induced cell death was exacerbated (Fig. 4A), indicating that OPC exerts its protective effects via basal Nrf2. The results shown in Fig. 2A and C demonstrated that the mRNA and protein expression levels of HO-1, NQO1 and TXNRD1 were significantly increased following treatment with OPC. However, the antioxidant activity was almost abolished with Nrf2 knockout (Fig. 4D-F). OPC was almost ineffective against H₂O₂-mediated SOD, CAT and GSH-Px activity when Nrf2 was knocked out (Fig. 4D-F). This finding suggested that OPC protected the A549 cells against H₂O₂-induced oxidative stress via the Nrf2-ARE pathway.

Discussion

The increased production of ROS (22,23) is frequently observed in cancer cells compared to normal cells, and this may be selectively used to promote tumor cell death. Cellular exposure to xenobiotics, drugs, heavy metals and ionizing radiation is able to generate ROS that leads to oxidative stress and has a profound impact on the survival and evolution of all living organisms (24). OPC, as a natural compound, has high bioactivity and provides significantly greater protection against free radicals, and free radical-induced lipid peroxidation and DNA damage than vitamins C and E, and β -carotene both in *in vitro* and *in vivo* models (25). In this study, we induced oxidative stress in A549 cells using H₂O₂ to uncover the underlying mechanisms of H₂O₂-induced oxida-

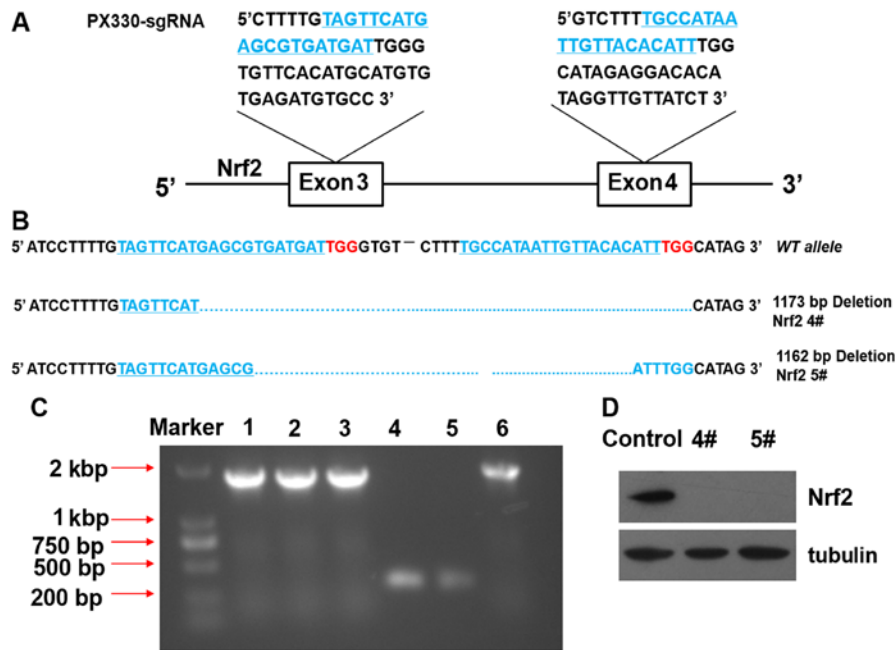


Figure 3. Generation of NF-E2-related factor 2 (Nrf2) knockout A549 cells using the CRISPR/Cas9 system. (A) The sgRNA (blue) was designed to aim at 20 pairs of bases in exon 3 and exon 4 of Nrf2. (B) Sequences of the wild-type (WT) Nrf2 locus and DSBs induced by Cas9 of Nrf2 locus in 2 established A549 cell lines (Nrf2-KO 4#) 4# and (Nrf2-KO 5#) 5#. Lanes 1, 2, 3 and 6 shown an allele of approximately 1,500 bp. The 4# and 5# cell lines had one allele with >1,100 bp deletion. The PAM sequence is indicated by red color. (C) Allele-specific PCR analysis of Nrf2 genome in A549 cells. (D) Western blot analysis of Nrf2 in A549, 4# and 5# cells.

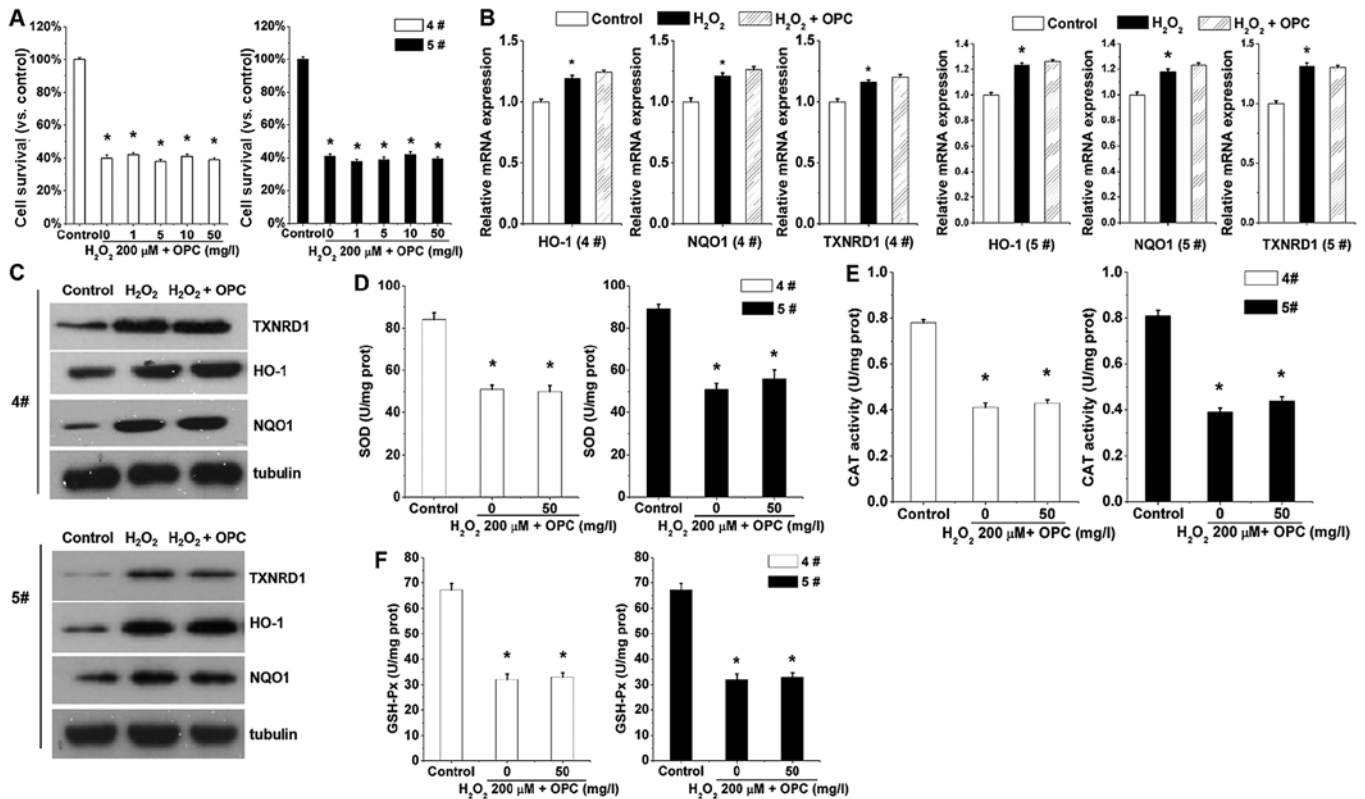


Figure 4. NF-E2-related factor 2 (Nrf2) mediates oligomeric proanthocyanidins (OPC)-induced antioxidant activity. (A) Effect of OPC on cell viability in 4# and 5# cells. Cells were pre-treated with 1-50 mg/l OPC 5 h and then exposed to 200 μM H₂O₂ for 12 h. Results are expressed as % viability of the control. Data are expressed as the means ± SD (for each group n=6). *p<0.05 vs. control group. (B) 4# and 5# cells were exposed to 200 μM H₂O₂ for 12 h with or without treatment with 50 mg/l OPC for 5 h. RNA was extracted and RT-qPCR was performed. Values were normalized to actin, and controls were set equal to 1. *p<0.05 vs. control group. (C) 4# and 5# cells were exposed to 200 μM H₂O₂ for 12 h with or without 50 mg/l OPC. Cell lysates were collected at 5 h post-treatment and subjected to western blot analysis using NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase-1 (HO-1) and thioredoxin reductase 1 (TXNRD1) antibodies. (D-F) Effects of OPC on superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) level in H₂O₂-exposed 4# and 5# cells. Cells were pre-treated with 50mg/l OPC for 5 h and then exposed to 200 μM of H₂O₂ for 12 h. Data are expressed as the means ± SD (n=6). *p<0.05 vs. control group.

tive toxicity. Cells have internal antioxidant defense enzymes, such as GSH-Px, CAT and SOD, which are critical protective measures against oxidative stress-related disorders. It has been previously suggested that antioxidants protect cells against oxidative damage by reducing lipid peroxidation products and elevating the levels of GSH-Px and SOD activities (26). In this study, the activities of these antioxidant defense enzymes were observed to be significantly reduced in A549 cells following exposure to H_2O_2 , and pre-treatment of the cells with OPC for 5 h significantly attenuated the effects of H_2O_2 on these enzymes, thereby boosting the protective effects of the enzymes for the cells (Fig. 1D-F).

Nrf2 is a transcription factor that is activated by increased ROS production, which induces the transcription of several antioxidant and detoxification enzymes, including HO-1, NQO1 and TXNRD1 (27). In this study, we present our findings that OPC is able to upregulate the Nrf2 signaling pathway under conditions of H_2O_2 -induced oxidative stress by enhancing the activation of ARE. Based on the molecular data obtained, we used CRISPR/gRNA technology to knockout Nrf2 and demonstrated that OPC is able to upregulate the Nrf2-dependent antioxidant response under conditions of H_2O_2 -induced oxidative stress. Previous studies have shown that an advantage of the constitutive activation of Nrf2 for cancer cells is the enhancement of proliferation. This enhanced proliferation enhances the ability of the cancer cells to detoxify the elevated levels of ROS associated with constant division and growth (28,29).

In conclusion, the results obtained in this study demonstrate that the protective effects of OPC against H_2O_2 -induced oxidative stress are dependent on the enhanced activity of Nrf2, which facilitates cells viability even in a highly oxidative environment.

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