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# Fucoxanthin Enhances HO-1 and NQO1 Expression in Murine Hepatic BNL CL.2 Cells through Activation of the Nrf2/ARE System Partially by Its Pro-oxidant Activity

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**ABSTRACT:** To determine whether fucoxanthin, a major carotenoid in brown sea algae, may activate cellular antioxidant enzymes via up-regulation of the Nrf2/antioxidant-response element (ARE) pathway, we incubated mouse hepatic BNL CL.2 cells with fucoxanthin (0.5−20 $\mu$M) for 0−24 h. We found that fucoxanthin (≥5 $\mu$M) significantly increased cellular reactive oxygen species (ROS) at 6 h of incubation, whereas preincubation with $\alpha$-*d*-tocopherol (30 $\mu$M) significantly attenuated the increase of ROS, indicating the pro-oxidant nature of fucoxanthin. Fucoxanthin significantly increased the phosphorylation of ERK and p38 and markedly increased nuclear Nrf2 protein accumulation after incubation for 12 h. Moreover, fucoxanthin significantly enhanced binding activities of nuclear Nrf2 with ARE and increased mRNA and protein expression of HO-1 and NQO1 after incubation for 12 h. siRNA inhibition of Nrf2 led to markedly decreased HO-1 and NQO1 protein expression. Thus, fucoxanthin may exert its antioxidant activity, at least partly, through its pro-oxidant actions.

**KEYWORDS:** Fucoxanthin, HO-1, NQO1, Nrf2/ARE, pro-oxidant activity

## INTRODUCTION

Oxidative stress is known to act as a predisposing factor to multistage carcinogens. In response to oxidative stress, cellular protection against oxidative and electrophile toxicities is provided by two types of antioxidant activities.[1−3] One is direct antioxidant activity, which scavenges reactive oxygen and nitrogen intermediates directly and instantaneously, and the other is indirect antioxidant activity, which enhances the expression of antioxidant enzymes and cytoprotective proteins primarily through the activation of nuclear factor-erythoid 2 related factor 2 (Nrf2) signaling. Much evidence indicates that dietary polyphenols and other classes of phytochemicals can induce the expression of cellular defense genes, such as phase II biotransferase and stress response proteins.[4,5] The induction of phase II biotransferase/antioxidant enzymes that protect against electrophilic and reactive oxygen species (ROS) is a potential major strategy in reducing the risk of cancer and other chronic degenerative diseases.[6]

Nrf2, a redox-sensitive transcription factor, is inactivated in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) under normal conditions. Keap1-mediated ubiquitination and proteasomal degradation of Nrf2 have been proven to be the primary control in turning on or off the Nrf2 signal.[7] Under normal conditions, a low basal level of Nrf2 is achieved by constant degradation through the Keap1-Cul3-Rbx1-dependent ubiquitination and proteasomal degradation.[8,9] When cells are under oxidative stress, Keap1 is easily oxidized because of its cysteine groups, resulting in decreased affinity for Nrf2,[10] which allows the translocation of Nrf2 to the nucleus and the binding to antioxidant response elements (ARE),[11] leading to enhanced expression of many stress-induced cytoprotective proteins, including HO-1 and the phase II biotransferase, such as NAD(P)H: quinine oxidoreductase 1 (NQO1), glutathione S-transferase (GST).[12,13] Previous reports have revealed that some dietary compounds, such as sulforaphane,[13] curcumin,[14] and epigallocatechin-3-gallate (EGCG),[15] can induce Nrf2-ARE mediated gene expression, which is one of the mechanisms underlying their chemopreventive effects.

Fucoxanthin is the major nonprovitamin A carotenoid found in *U. pinnatifida* and has been shown to have multiple biological functions, such as antioxidant activity,[16−18] induction of apoptosis[19] and cell cycle arrest,[20] and antiobesity.[21] The antioxidant activities of fucoxanthin in cell-free systems include scavenging of hydroxyl radical, superoxide radical, singlet oxygen,[16] DPPH radical, 12-doxyl-stearic acid (12DS), and nitrobenzene with linoleic acid (NB-L) scavenging activity.[22] Moreover, cell studies indicate that fucoxanthin inhibits oxidative damage in monkey kidney fibroblast cells and human fibroblast cells induced by $H_2O_2$ and ultraviolet B.[17,18] An in vivo study indicated that administration of fucoxanthin suppresses the lipid peroxidation induced by retinol deficiency possibly by modulating catalase, GST, and $Na^+$/ $K^+$-ATPase activities.[23] However, it is unclear whether fucoxanthin induces antioxidant enzymes and/or cytoprotective proteins through the Nrf2/ARE pathway. In the present study, we used BNL CL.2 cells as the cell model based on a published report to determine whether fucoxanthin may activate HO-1 and NQO1 expression via up-regulation of the Nrf2/ARE pathway.[24] We employed luciferase reporter gene assays to determine the binding activity of Nrf2 to ARE. In addition, we used small interfering RNA inhibition of Nrf2 in BNL CL.2 cells to confirm the activation of Nrf2/ARE by fucoxanthin.

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## MATERIALS AND METHODS

**Chemicals and Purification of Fucoxanthin.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, sodium pyruvate, and nonessential amino acids (NEAA) were from Gibco/BRL (MD). Anti-Nrf2 mouse monoclonal antibody was purchased from Sigma Co. (St. Louis, MO), MAPK/extracellular signal-regulated kinase (ERK) 1/2, and p38 MAPK proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). Anti-NQO1 and anti-HO-1 mouse monoclonal antibodies were purchased from Epitomics. Fucoxanthin was extracted from *Undaria pinnatifida* and purified as we reported previously.[25] Dried powder of *U. pinnatifida* purchased from a market was first soaked in two volumes (v/w) of acetone for one night. This extraction was repeated twice. Water/*n*-hexane/ethanol (1:1:2, v/v/v) solution was added to these extracts, and then, the lower layer was obtained with a separatory funnel. The fucoxanthin fraction orange-colored was separated by means of silica gel column chromatography developed with acetone/*n*-hexane (3:7, v/v). Purification of fucoxanthin was finally carried out by reversed phase high-performance liquid chromatography (HPLC). HPLC experiments were carried out with a Hitachi HPLC-D7000 equipped with photodiode array detector. The column used was ODS UG-5 (250 mm × The flow rate was 1.0 mL/min. The detector was set at 450 nm for detecting fucoxanthin. The purity of fucoxanthin obtained as described above was 99.2%. The purified fucoxanthin was dissolved in ethanol to a final concentration of 10 mM. Before the experiment, fucoxanthin solution was diluted in a mixture of ethanol and FBS (1:9), as adopted from the preparation of lycopene solution.[26] The diluted solution was directly used for cell incubation, and the final concentrations of fucoxanthin in the culture medium were 1−10 $\mu$M. The final concentrations of ethanol and FBS in the culture medium were 0.2 and 1.8%, respectively, and ethanol at this concentration did not affect cell viability.

**Cell Culture.** The murine embryonic liver BNL CL.2 cells (BCRC 60180) was purchased from Food Industry Research and Development Institute (Hsin Chu, Taiwan) and were cultured in DMEM (Invitrogen) containing 10% (v:v) FBS (Invitrogen), 0.37% (w/v) $NaHCO_3$, penicillin (100 kU/L), and streptomycin (100 kU/L) in a humidified incubator under 5% $CO_2$ and 95% air at 37 °C.

**Extraction of Nuclear Proteins.** The BNL CL.2 cells were washed twice in phosphate-buffered saline (PBS), harvested by trypsinization, and suspended in 1 mL of PBS, followed by centrifugation (800*g* for 3 min at 4 °C). The cell pellet was carefully resuspended in 200 $\mu$L of cold buffer A, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min to allow cells to swell. After this time, 15 $\mu$L of 10% Nonidet P-40 was added, and the tube was vortex-mixed for 10 s. The homogenate was then centrifuged at 800*g* for 3 min at 4 °C. The resulting nuclear pellet was resuspended in 30 $\mu$L of cold buffer B, consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 $\mu$M dithiothreitol, and protease inhibitors. The pellet was then incubated on ice for 15 min and vortex-mixed for 10−15 s every 2 min. The nuclear extract was finally centrifuged at 15000*g* for 5 min at 4 °C. The supernatant containing the nuclear proteins was stored at −80 °C.

**Western Blotting.** Protein expression of Nrf2, NQO1, HO-1, ERK, p38, JNK, p-JNK, p-ERK, and p-p38 was measured by Western blotting. In cell culture experiments, the medium was removed, and cells were rinsed with PBS twice. After the addition of 0.5 mL of cold RIPA buffer and protease inhibitors, cells were scraped followed by a vortex at 0 °C for 20 min. The cell lysates were then subjected to a centrifugation of 10000*g* for 30 min at 4 °C. The protein (50 $\mu$g) from the supernatant was resolved on SDS-PAGE and transferred onto a PVDF membrane (Millipore). After blocking with TBS buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) containing 5% nonfat milk, the membrane was incubated with monoclonal antibody followed by horseradish peroxidase-conjugated antimouse IgG (Santa Cruz, CA) and then visualized using an ECL chemiluminescent detection kit (Amersham). The relative density of the immunoreactive bands was quantitated by densitometry (Gel Pro Analyzer TM, version 3.0, Media Cybernetics, United States).

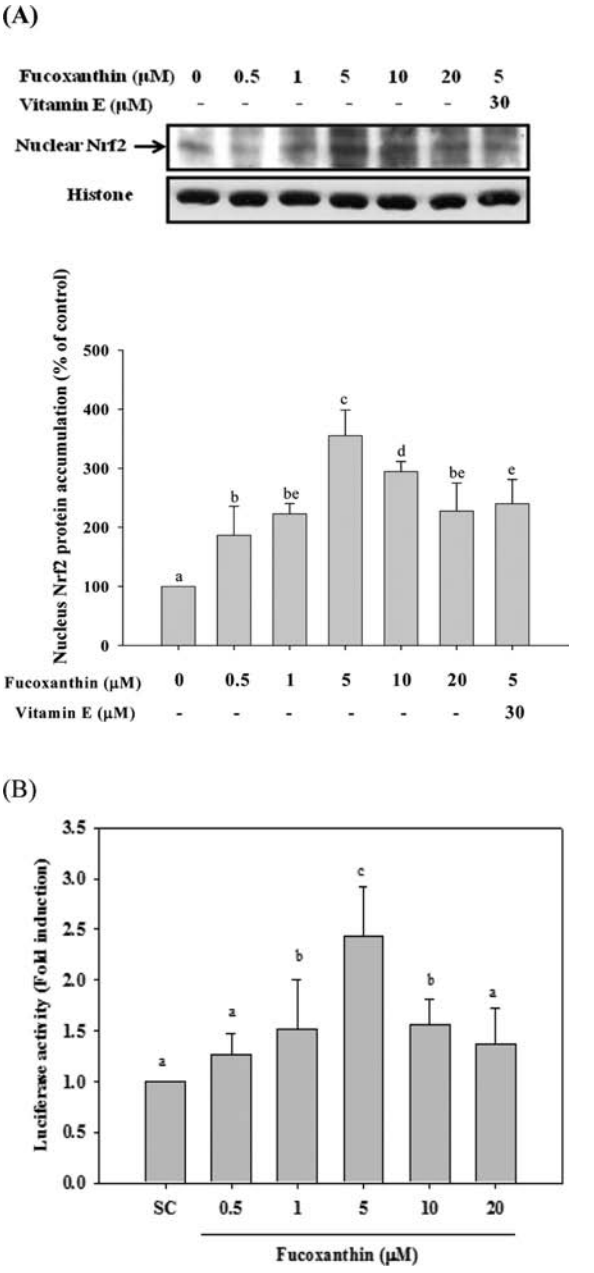


**Figure 1.** Effects of either fucoxanthin (0−20 $\mu$M) or fucixanthin (5 $\mu$M) plus vitamin E (30 $\mu$M) on nuclear Nrf2 protein accumulation and Nrf2/ARE binding activity determined by the luciferase reporter gene assay in BNL CL.2 cells. (A) Western blots of nuclear Nrf2 and $\beta$-actin. (B) Nrf2/ARE binding activity. Values are means ± SDs, $n$ = 3; means without a common letter differ significantly ($P$ < 0.05).

**Reverse-Transcription Polymerase Chain Reaction (RT-PCR) of HO-1 and NQO1.** The total RNA in cell cultures was extracted with REzol reagent (Protech), and 1 $\mu$g of total RNA was reverse-transcribed by using oligo-dT as a primer in 20 $\mu$L reverse-transcription solutions (Promega, United States). The RT-PCR conditions for *HO-1*

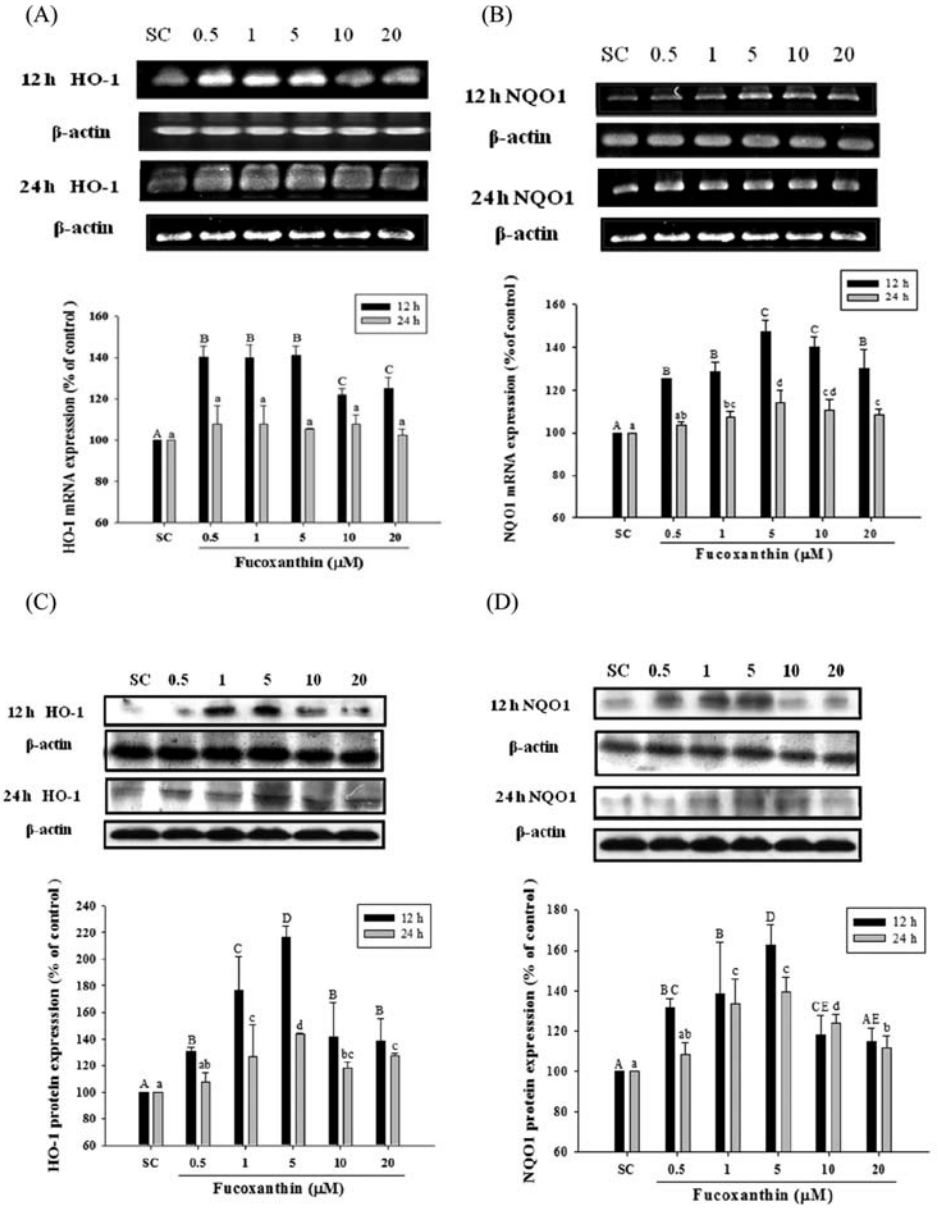


**Figure 2.** Effects of fucoxanthin on the HO-1 and NQO1 mRNA and protein expression in BNL CL.2 cells. Cells were incubated with various concentrations fucoxanthin (0−20 $\mu$M) for 12 and 24 h. (A) HO-1 mRNA expression. (B) NQO1 mRNA expression. (C) HO-1 protein expression (B) NQO1 protein expression. Values are means ± SDs, $n = 3$; means without a common letter at the same incubation time differ significantly ($P < 0.05$).

and *NQO1* were as follows: initial denaturation at 95 °C for 1 min, 1 min of annealing time (59 °C for *HO-1*, 61 °C for *NQO1*, and 60 °C for $\beta$-actin), and 1 min of amplification time for 40 cycles after an activation step of 2 min at 95 °C. PCR products were analyzed by electrophoresis on 1% agarose gel. The primers used in this study were as follows: *HO-1* forward, 5′-ACATCGACAGCCCCACCAAGTTCAA-3′; reverse, 5′-CTGACGAAGTGACGCCATCTGTGAG-3′; *NQO1* forward, 5′-CCATTCTGAAAGGCTGGTTTG-3′; reverse, 5′-CTAGCTTTGATCTGGTTGTC-3′; and $\beta$-actin forward, 5′-GTGGGGCGCCCCAGGCACCA-3′; reverse, 5′-CACCCCGCGGGGTCCGTGGT-3′.

**Assay of Intracellular ROS.** The redox-sensitive fluorescent probe 2′,7′-dichlorofluorescenin diacetate (DCFH-DA) was used to assess the intracellular level of ROS.[27] BNL CL.2 cells were seeded onto six-well culture plates and incubated with fucoxanthin for 1−12 h. After the flash medium was replaced, cells were incubated with 10 $\mu$M DCFDA (in methanol; final concentration, 0.2%) for 30 min, and intracellular ROS were determined using flow cytometry with the data being analyzed using MODFIT ROS analysis program (FACS Calibur, BD, United States).

**Recombinant Plasmid and Luciferase Reporter Gene Assays.** To construct p3xARE/Luc, tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site, 5′TGACTCAGCA-3′,[28] were introduced into the restriction sites of pGL3 promoter plasmid (Promega Corp.). Transfection of p3xARE/Luc-pGL3 into BNL CL.2 cells was performed using TransIL-LT1 Transfection Reagent (Mirus), and in all experiments, the pRL-TK Renilla reporter vector (Promega) was used as an internal control. Renilla and firefly luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

**Nrf2 RNA Interference Assay.** The siRNA sequence targeting Nrf2 corresponds to the coding region nucleotides 1903−1921 (5′-GTAAGAAGCCAGATGTTAA-3′) in the Nrf2 cDNA. The Nrf2 siRNA duplex with the following sense and antisense sequences was

**Table 1. Levels of Intracellular ROS in BNL CL.2 Cells Incubated Either Fucoxanthin or Vitamin E Plus Fucoxanthin for 1−12 h**

| | mean DCF fluorescence intensity[a] | | | |
|---|---|---|---|---|
| treatments | 1 h | 3 h | 6 h | 12 h |
| solvent control | 6 ± 3 | 12 ± 2[a] | 8 ± 3[a] | 11 ± 2[a] |
| 0.5 μM fucoxanthin | 5 ± 1 | 14 ± 1[a] | 10 ± 2[a] | 12 ± 2[ab] |
| 1 μM fucoxanthin | 5 ± 2 | 12 ± 2[a] | 12 ± 2[a] | 12 ± 2[ab] |
| 5 μM fucoxanthin | 6 ± 1 | 13 ± 3[a] | 21 ± 5[b] | 12 ± 2[ab] |
| 10 μM fucoxanthin | 6 ± 1 | 13 ± 4[a] | 24 ± 3[b] | 12 ± 2[ab] |
| 20 μM fucoxanthin | 6 ± 2 | 23 ± 8[b] | 34 ± 5[c] | 13 ± 2[b] |
| 5 μM fucoxanthin + 30 μM vitamin E[b] | 3 ± 1 | 8 ± 1[a] | 13 ± 2[a] | 9 ± 3[a] |
| 20 μM fucoxanthin + 30 μM vitamin E | 5 ± 1 | 11 ± 4[a] | 18 ± 3[b] | 11 ± 3[ab] |
| 1 mM $H_2O_2$[c] | 87 ± 6 | 83 ± 15 | 91 ± 7 | 98 ± 1 |

[a] Intracellular levels of ROS were measured using 2′,7′-dichlorofluorescenin diacetate (DCFF-DA) and expressed as DCF fluorescence intensity. Values are means ± SDs, $n = 3$; means in the same column (except for $H_2O_2$) without a common letter differ significantly ($P < 0.05$). [b] The cells were preincubated with 30 μM vitamin E for 1 h followed by incubation with fucoxanthin for 1−12 h. [c] The cells were incubated with 1 mM hydrogen peroxide at 37 °C for 30 min before the DCFH-DA assay.

used, 5′-GUAAGAAGCCAGAUGUUAAdUdU-3′ (sense) and 3′-dU-dUCAUUCUUCGGUCUACAATT-5′ (antisense). To confirm the specificity of the inhibition, the siCONTROL nontargeting siRNA (5′-UAGCGACUAAACACAUCAAUU-3′) was used as a negative control. All of the siRNA duplexes were synthesized by Dharmacon Research (Lafayette, CO). Cells were transfected siRNA (70 nM) for 48 h using Lipofectamine 2000 and OPTI-MEM reduced serum medium (Invitrogen) according to the manufacturer's recommendations.

**Statistical Analysis.** Values are expressed as means ± SDs and analyzed using one-way analysis of variance followed by least significant differences test for comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a $P$ value <0.05 is considered statistically significant.

## RESULTS

**Fucoxanthin Increases Nuclear Nrf2 Protein Accumulation and ARE-Luciferase Activity.** Incubation of BNL CL.2 cells with fucoxanthin (0.5−5 μM) for 12 h significantly and concentration dependently increased nuclear Nrf2 protein accumulation by 255%, $P < 0.05$, at 5 μM fucoxanthin) (Figure 1A). By contrast, the effect of fucoxanthin at 10 and 20 μM on Nrf2 was lower than that of fucoxanthin added at 5 μM (by 194% for 10 μM and 128% for 20 μM fucoxanthin). Treatment of BNL CL.2 with fucoxanthin (5 μM) in combination with vitamin E (30 μM) for 12 h significantly decreased nuclear Nrf2 accumulation by 32% ($P < 0.05$), as compared with 5 μM fucoxanthin alone. These results suggest that fucoxanthin promotes cytoplasmic Nrf2 to translocate to nucleus via pro-oxidative actions. In addition, fucoxanthin significantly and concentration-dependently increased ARE-luciferase activity at 12 h of incubation (by 143%, $P < 0.05$, at 5 μM fucoxanthin) (Figure 1B), although the effects of fucoxanthin at 10 and 20 μM were lower than that at 5 μM. The results indicate that fucoxanthin enhanced the binding activities between nuclear Nrf2 and ARE.

**Fucoxanthin Increases HO-1 and NQO1 at Both mRNA and Protein Levels.** Treatment of BNL CL.2 with fucoxanthin (0.5−5 μM) for 12 h significantly increased *HO-1* and *NQO1* mRNA expression in a concentration-dependent manner (by 41 and 47%, $P < 0.05$, at 5 μM fucoxanthin, respectively), whereas the effects of fucoxanthin at 10 and 20 μM were lower than that at 5 μM (Figure 2A,B). In contrast, fucoxanthin did not significantly affect *HO-1* and *NQO1* mRNA expression at 24 h of incubation. We then analyzed the level of HO-1 and NQO1 protein expression, and we found that the expression of HO-1 and NQO1 protein also increased by fucoxanthin (0.5−5 μM) at 12 h of incubation (by 116% for HO-1 and 62% for NQO1, $P < 0.05$, at 5 μM fucoxanthin), although the effect of fucoxanthin at 10 and 20 μM was somewhat lower than that of 5 μM (Figure 2C,D). The results indicate that fucoxanthin increases HO-1 and NQO1 at both the mRNA and the protein levels.

**Fucoxanthin Increases Intracellular ROS in BNL CL.2 Cells.** Intracellular ROS were determined using the dichlorofluorescein assay. We found that fucoxanthin treatment (0.5−20 μM) for 1 and 3 h did not increase the intracellular ROS. After incubation for 6 h, fucoxanthin added at 5−20 μM, but not at 0.5 and 1 μM, significantly increases endogenous ROS generation (Table 1). In addition, treatment of BNL CL.2 with fucoxanthin (5 μM) in combination with vitamin E (30 μM) for 6 h decreased the intracellular ROS (by 38%, $P < 0.05$), as compared with 5 μM fucoxanthin alone. However, levels of intracellular ROS induced by fucoxanthin returned to the control level at 12 h of incubation. The results indicated that fucoxanthin was pro-oxidative in cultured cells.

**Fucoxanthin Increases the Phosphorylation of ERK and p38.** The effect of fucoxanthin on protein expression of the MAPK family (p38, ERK, and JNK) in BNL CL.2 cells was determined by Western blotting (Figure 3). Results revealed that fucoxanthin (5 μM) markedly increased the phosphorylation of ERK and p38 at 12 h of incubation, but it did not affect the phosphorylation of JNK (Figure 3A) and the protein of ERK, p38. We then determined the concentration effects of fucoxanthin (0.5−20 μM) at 12 h of incubation on the phosphorylation of p38 and ERK in BNL CL.2 cells. We found that fucoxanthin increased the phosphorylation of ERK and p38, and the effect was concentration-dependent up to 5 μM, whereas the effect of fucoxanthin at 10 and 20 μM was lower than that at 5 μM (Figure 3B). We further examined whether fucoxanthin increases the phosphorylation of MAPK family through elevated intracellular ROS. We found that treatment of BNL CL.2 with fucoxanthin (5 μM) in combination with vitamin E (30 μM) for 12 h significantly decreased the protein expression of p-ERK, p-p38 (by 80 and 43% $P < 0.05$, respectively), as compared with 5 μM

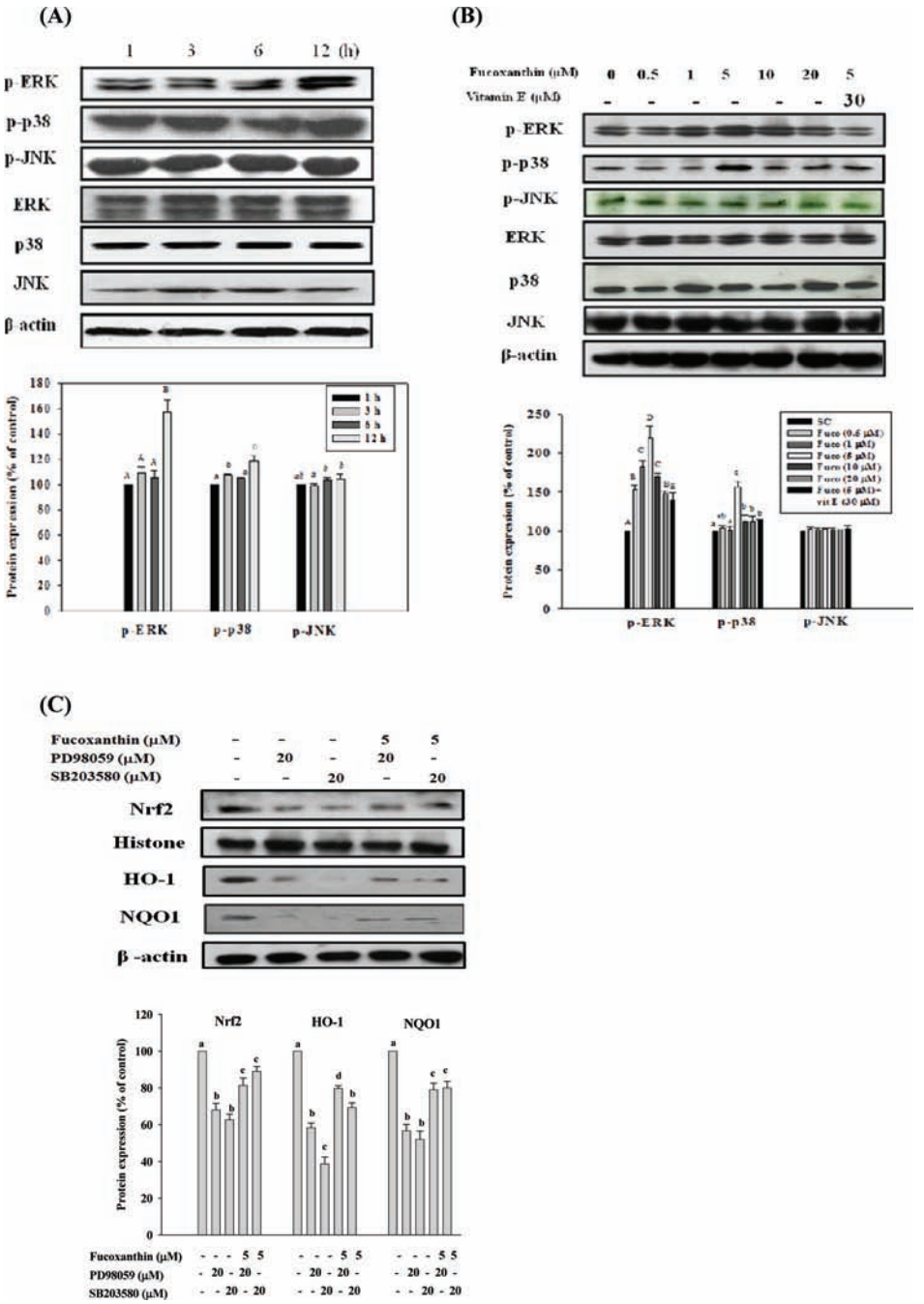


**Figure 3.** Effects of fucoxanthin on protein expression of the MAPK family (p38, ERK, and JNK) and HO-1 and NQO1 as well as on protein accumulation of Nrf2 after treatment with an ERK inhibitor (PD98059) or a p38 inhibitor (SB203580) in BNL CL.2 cells. (A) Cells were incubated with 10 $\mu$M fucoxanthin for 1−12 h. (B) Cells were incubated with various concentrations (0−20 $\mu$M) of fucoxanthin for 12 h. (C) Cells were pretreated with PD98059 (20 $\mu$M) or SB203580 (20 $\mu$M) for 1 h and then incubated with fucoxanthin (5 $\mu$M) for 24 h. Values are means ± SDs, $n$ = 3; means without a common letter at the same incubation time differ significantly ($P < 0.05$).

fucoxanthin alone, suggesting that the increased phosphorylation of MAPK by fucoxanthin is related to the pro-oxidant activity of this carotenoid.

To further determine whether the effects of fucoxanthin on activation of HO-1 and NQO1 expression occur primarily through ERK/p38 pathway, BNL CL.2 cells were pretreated with an ERK inhibitor (PD98059, 20 $\mu$M) or a p38 inhibitor (SB203580, 20 $\mu$M) for 1 h and then incubated with fucoxanthin (5 $\mu$M) for 12 h. This concentration of fucoxanthin (5 $\mu$M) was chosen because it produced a strong activation of HO-1 and NQO1 expression (Figure 2). In contrast, treatment with each of the two inhibitors markedly decreased the protein expression of HO-1 and NQO1. The combined treatment of fucoxanthin with a MAPK inhibitor produced significant activations of HO-1 and NQO1 expression, as compared with the MAPK inhibitor alone (Figure 3C). Thus, the data indicate that fucoxanthin induces antioxidant and cytoprotective enzymes and that this effect involves the activation of the ERK/p38 pathway.

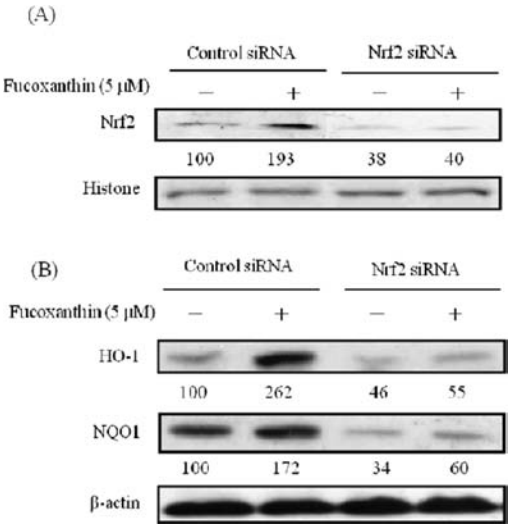


**Figure 4.** Effects of fucoxanthin on antioxidant enzymes and phase II biotransferases in BNL CL.2 cells via the Nrf2/ARE pathway. Cells were transiently transfected with Nrf2 siRNA (70 nM) for 48 h, and the protein levels of Nrf2, HO-1, and NQO1 were determined by Western blotting.

**Effects of Silencing Nrf2 Expression in BNL CL.2 Cells.** To further demonstrate that fucoxanthin activates the Nrf2/ARE system to induce HO-1 and NQO1 gene expression, we used small interfering RNA inhibition of Nrf2 on BNL CL.2 cells at 48 h of incubation. We found that Nrf2 siRNA treatment decreased the protein expression of Nrf2, HO-1, and NQO1 (by 62, 54, and 66%, respectively), as compared with control siRNA (Figure 4). Thus, the data indicate that fucoxanthin induces antioxidant and cytoprotective enzymes and that this effect involves the activation of the Nrf2/ARE pathway.

## DISCUSSION

Fucoxanthin has been shown to exhibit direct antioxidant activities in vitro and to modulate catalase, GST, and $Na^+/K^+$-ATPase to suppress lipid peroxidation.[23] The main question addressed by this study was whether fucoxanthin induces cytoprotective proteins through Nrf2/ARE pathway. In the present study, we demonstrate for the first time that fucoxanthin induced both the nuclear accumulation of Nrf2 protein and the induction antioxidant enzymes and phase II biotransferase including HO-1, NQO1 in murine embryonic hepatic BNL CL.2 cells.

A probable mechanism by which fucoxanthin activates Nrf2/ARE is through the pro-oxidative activity of fucoxanthin. A pro-oxidant or an electrophile can modify cysteine residues of Keap1, which results in a conformational change in Keap1 and the inability of Cul3 to ubiquitinate Nrf2 and then allows Nrf2 to translocate into nucleus.[2] Previous reports have revealed that the activation of Nrf2-ARE signaling by antioxidative phytochemicals to induce cytoprotective enzymes may, at least in part, be due to their pro-oxidant activity.[2] In the present study, we found that fucoxanthin (1−20 $\mu$M) significantly increased intracellular ROS generation at 6 h of incubation and that treatment of BNL CL.2 with fucoxanthin in combination with vitamin E markedly decreased intracellular ROS, indicating that fucoxanthin has pro-oxidative properties. The pro-oxidant activity of fucoxanthin may arise from the 5,6-monoepoxide, a unique structure of fucoxanthin, which has been shown to undergo ring-opening reactions

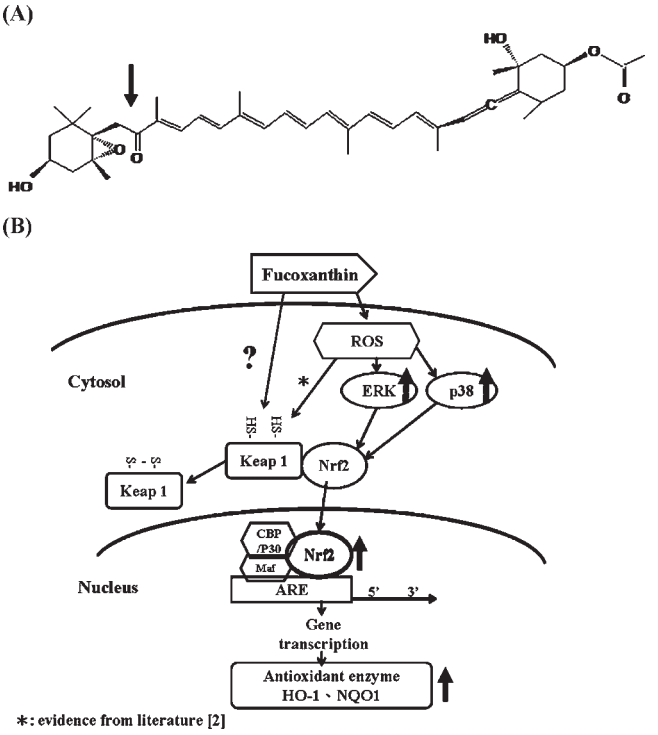


**Figure 5.** (A) Chemical structure of fucoxanthin. (B) A proposed mechanism of fucoxanthin-activated antioxidant enzymes and phase II biotransferases through the Nrf2-ARE pathway in BNL CL.2 cells. The scheme highlights the signaling steps identified in the present study. The asterisk is based on evidence form ref 2; the question mark represents an unconfirmed pathway.

as a result of attacking nucleophiles.[29] Consequently, ROS produced by fucoxanthin may activate the upstream protein kinases, such as MAPK pathway to induce Nrf2 phosphorylation,[30] resulting in the dissociation of Nrf2 from its repressor keap1 and its translocation into nucleus.[31,32] Indeed, we found that fucoxanthin significantly increased the phosphorylation of ERK and p38, leading to enhanced nuclear translocation of Nrf2 and expression of HO-1 and NQO1.

However, the pro-oxidant activity of fucoxanthin may not fully account for the activation of Nrf2 in BNL CL.2 cells because coincubation of fucoxanthin with vitamin E only partially attenuated the activation of Nrf2, indicating that additional signaling pathways may be involved. For instance, fucoxanthin may cause Nrf2 translocation by reacting with the thiol groups of Keap 1 through Michael addition because of the presence of an $\alpha,\beta$-unsaturated carbonyl group in its structure (Figure 5A). In this respect, curcumin, which contains two $\alpha,\beta$-unsaturated carbonyl groups in its structure, is known to induce cytoprotective effects through the Nrf2/ARE pathway by Michael addition, whereas tetrahydrocurcumin, which lacks an $\alpha,\beta$-unsaturated carbonyl group, fails to induce the cytoprotective proteins through the Nrf2/ARE pathway.[33]

In this study, we noted that the concentration effects of fucoxanthin on all experiments were bell-shaped; that is, the effects of fucoxanthin at concentrations $\geq$10 $\mu$M were less effective than those at 5 $\mu$M. Consistent with these findings, carotenoids including $\beta$-carotene and lycopene were shown to have lowered effectiveness as antioxidants and anticarcinogens in vitro at concentrations >10 $\mu$M.[34,35] The reason for such a bell-shaped effect is not well understood.

In conclusion, the present study demonstrates that fucoxanthin significantly increases antioxidant enzymes and phase II biotransferase in BNL CL.2 cells and that this action is likely associated with pro-oxidative behavior of fucoxanthin, which leads to the translocation of Nrf2 through the activation of several upstream protein kinases. The pro-oxidative activity of fucoxanthin at concentration tested here (0.5−20 $\mu$M) may be considered to be mild, as it does not affect the viability of BNL CL.2 cells.[25] All of these data taken together, a possible chemopreventive mechanism of fucoxanthin has been established in the present study (Figure 5B).

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