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Apo-8′-lycopenal Induces Expression of HO-1 and NQO-1 via the ERK/p38-Nrf2-ARE Pathway in Human HepG2 Cells

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ABSTRACT: Lycopene and its metabolite apo-10′-lycopenoic acid have been shown to induce phase II detoxifying/antioxidant enzymes through activation of the nuclear factor erythroid-derived 2-like 2 (Nrf2)-antioxidant response element (ARE) transcription system. However, little is known about whether apo-8′-lyocpenal, one of the main metabolites of lycopene in rat livers, in lycopene-containing food, and in human plasma, has similar effects. This study investigated the effect of apo-8′-lycopenal on Nrf2-ARE system-mediated heme oxygenase 1 (HO-1) and NAD(P)H:quinine oxidoreductase 1 (NQO-1) expression in human HepG2 cells. It was found that apo-8′-lycopenal (1−10 μM) significantly increased nuclear Nrf2 accumulation, ARE-luciferase activity, Nrf2-ARE binding activity, chymotrypsin-like activity, and downstream HO-1 and NQO-1 expression, but decreased cytosolic Kelch-like ECH-associated protein 1 (Keap1) expression. Results also revealed that the ERK/p38-Nrf2 pathway is involved in activation of HO-1 and NQO-1 expression by apo-8′-lycopenal using Nrf2 siRNA and ERK/p38 specific inhibitors. In addition, the activation time of lycopene on nuclear Nrf2 accumulation is slower than that of apo-8′-lycopenal, suggesting that the chemopreventive effects of lycopene may be partially attributed to its metabolites.

KEYWORDS: *apo-8′-lycopenal, HepG2 cells, HO-1, NQO-1, Nrf2-ARE system*

INTRODUCTION

Cancer chemoprevention is a complex process that includes reversion, suppression, or prevention of carcinogenic processes by some natural, synthetic or biological compounds.[1] Nuclear factor erythroid-derived 2-like 2 (Nrf2), which is a member of the NF-E2 family of the basic leucine zipper of redox-sensitive transcription factors, has been shown to play a critical role in cancer chemoprevention.[2−5] Under normal physiological situations, Nrf2 is inactive in the cytoplasm by binding the skeletal actin-binding protein, Kelch-like ECH-associated protein 1 (Keap1), which suppresses the translocation of Nrf2 to the nucleus.[2] Keap1 is a molecular redox sensitive sensor due to the oxidation or modification of the Keap1 cysteine residues by reactive species followed by translocation of Nrf2 to the nucleus and binding to antioxidant response elements (ARE), leading to activation of phase II detoxifying/antioxidant enzymes including heme oxygenase 1 (HO-1), NAD(P)H:quinine oxidoreductase 1 (NQO1), and glutathione *S*-transferase (GST).[6−9] A large number of studies have indicated that some natural phytochemicals, such as sulforaphane,[10−13] curcumin,[14−18] epigallocatechin gallate (EGCG),[19−22] quercetin,[23−25] and resveratrol,[26,27] exert cancer chemopreventiive effects by induction of Nrf2-ARE system-mediated phase II detoxifying/antioxidant enzymes both in vitro and in vivo.

The roles of lycopene in cancer chemoprevention have been demonstrated in several preclinical and experimental studies, including antimetastasis both in highly invasive hepatocarcinoma and in SK-Hep-1 cell-bearing nude mice,[28−30] antiangiogenesis,[30,31] antiproliferation and induction of apoptosis in human colon carcinoma,[32] human erythroleukemia[32] and human prostate cancer cells,[33,34] and transactivation of the nuclear receptors superfamily.[35] The antioxidant activities of lycopene have been considered to be the major contributors for its chemopreventive effects.[36] In human breast cancer MCF-7 cells and human hepatoma HepG2 cells, lycopene has been shown to activate the expression of γ-glutamylcysteine synthetase and NQO-1 through the Nrf2-ARE transcriptional system.[37] In addition, activation of phase II detoxifying/antioxidant enzymes, such as glutathione reductase and GST, is considered to be one of the anticarcinogenic mechanisms of lycopene.[7,38]

Several studies have indicated that the chemopreventive activity of lycopene may be attributed, at least in part, to lycopene metabolites.[39−42] Oxidative/metabolic products of lycopene, but not lycopene itself, are the activators in the mediation of the Nrf2-ARE transcriptional system.[43] Lycopene can be cleaved by carotene-oxygenase to form apo-lycopenal, apo-lycopenol, and apo-lycopenoic acid.[41] In the rat liver, lycopene is mainly metabolized by carotenoid monoxygenase II into apo-8′-lycopenal and apo-12′-lycopenal.[44] A recent study has demonstrated that apo-12′-lycopenal inhibits the proliferation of prostate cancer cells.[39] Notably, an enzymatic metabolite of lycopene, apo-10′-lycopenoic acid, has been shown to activate Nrf2-mediated expression of HO-1, NQO-1, and GST in human bronchial epithelial cells[41] and to suppress lung cancer cell growth both in vitro and in vivo.[40] In addition, the levels of apo-8′-lycopenal in lycopene-containing food and in the plasma of the general population after consumption of a diet high in tomato juice for 8 weeks are higher than those of other metabolites including apo-6′-, apo-10′-, and apo-14′-lycopenal.[45]

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Lycopene and it enzymatic metabolite apo-10′-lycopenoic acid have been shown to induce the expression of phase II detoxifying/antioxidant enzymes through activation of the Nrf2-ARE transcriptional system.[37,41] However, little is known of whether apo-8′-lycopenal, one of the major metabolites of lycopene in rat livers, in lycopene-containing food and in human plasma, has similar effects. In the present study, we investigated the role of apo-8′-lycopenal on nuclear Nrf2 accumulation in comparison with lycopene and on the expression of HO-1 and NQO-1 using Western blotting and reverse transcription PCR in human HepG2 cells. The human hepatoma HepG2 cell line has been widely used and is considered to be one of the experimental models for biochemical and nutritional studies.[46,47] Moreover, this transformed cell line is well-differentiated and closely resembles the human hepatocyte in culture,[48] rendering it suitable for all biochemical experiments in the present study. In addition, we employed the luciferase reporter gene assay and electrophoretic mobility shift assay (EMSA) to determine the effect of apo-8′-lycopenal on Nrf2-ARE binding activity. We further determined the role of the MAPK signaling pathway on activation of the Nrf2-ARE system by apo-8′-lycopenal using specific inhibitors of the MAPK family. Moreover, we used small interfering RNA inhibition of Nrf2 to confirm the activation of Nrf2-ARE-mediated HO-1 and NQO-1 expression by apo-8′-lycopenal in human HepG2 cells.

MATERIALS AND METHODS

Materials and Preparation of Apo-8′-lycopenal and Lycopene. The materials for cell culture such as Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, sodium pyruvate, and nonessential amino acids (NEAA) were purchased from GIBCO/BRL. The anti-Nrf2, anti-NQO1, and anti-HO-1 mouse monoclonal antibodies were from Epitomics, and phospho-MAPK family antibody sampler kits were from Cell Signaling Technology (Beverly, MA). Apo-8′-lycopenal was a gift from John W. Erdman, Jr. (Division of Nutritional Sciences, University of Illinois, Urbana, IL), and lycopene was purchased from Wako. The purity of apo-8′-lycopenal was >99%, as confirmed using HPLC in our laboratory, and the commercial lycopene is approximately 97%, as claimed by the supplier (Wako). Apo-8′-lycopenal or lycopene was dissolved in THF/BHT to form a 10 mM stock solution, which was diluted with THF at indicated ratios (1:1, 1:3, and 1:19) and then diluted with FBS at the indicated ratio (1:9).[49] THF/BHT-FBS-apo-8′-lycopenal or lycopene was added to the culture medium at a calculated final concentration of 1−10 μM. The final THF and FBS concentrations in medium were 0.2% (v/v) and 1.8%, which did not significantly affect the assays described below.

Cell Culture and Cell Proliferation Assay. The human hepatoma HepG2 cell line (BCRC No. 60025) was purchased from the Food Industry Research and Development Institute, Hsin Chu, Taiwan. HepG2 cells with passages between 15 and 19 were used in this study and were cultured in DMEM containing 10% (v/v) FBS, 0.37% (w/v) $NaHCO_3$, penicillin (100 U/mL), and streptomycin (100 U/mL) in a humidified incubator under 5% CO_2 and 95% air at 37 °C. For the cell proliferation assay, cells were cultured in 24-well plates at 2×10^4 cells/well for 24 h. Each well was washed with PBS, and then 1 mL of DMEM containing different concentration (1, 5, and 10 μM) of apo-8′-lycopenal or single concentration (10 μM) of lycopene was added for 12 and 24 h of incubation. After incubation, each well was washed twice with PBS followed by incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 h at 37 °C. The supernatant was removed, and then the formazan was dissolved in 1 mL of DMSO and measured spectrophotometrically at 570 nm. The percentage of viable cells was estimated by comparison with untreated control cells.

RNA Isolation and Reverse Transcription RCR. Total cellular RNA was extracted from HepG2 cell using REzol C&T reagent (Protech, Taiwan) and reverse transcribed into cDNA (MMLVReverse Transcriptase, Gibco/BRL) using oligo(dT) as primers and then amplified with primer bases on HO-1, NQO-1, and β-actin (internal control) sequences. The primers for amplifying HO-1 cDNA were 5′-ACATCGACAGCCCCACCAAGTTCAA-3′, located in the 5′-untranslated region, and 5′-CTGACGAAGTGACGCCATCTGTGAG-3′, located in the 3′-untranslated region. The primers for amplifying NQO-1 cDNA were 5′-CCATTCTGAAAGGCTGGTTTG-3′, located in the 5′-untranslated region, and 5′-CTAGCTTT-GATCTGGTTGTC-3′, located in the 3′-untranslated region. The primers for amplifying β-actin cDNA were 5′-GTGGGGCGCCCCAGGCACCA-3′, located in the 5′-untranslated region, and 5′- CACCCCGCGGGGTCCGTGGT-3′, located in the 3′-untranslated region. PCR amplification was performed with a thermal cycler as follows: denaturation at 95 °C for 30 s, annealing at 59 °C for HO-1, at 61 °C for NQO1, or at 60 °C for β-actin for 30 s, extension at 72 °C for 90 s (40 cycles), followed by a final incubation at 72 °C for 7 min. The PCR products were added to 6× staining buffer (EZVision Three DNA Dye & Buffer, Amresco) and were subjected to 1% agarose gel electrophoresis. Matrox Inspector 2.1 software was used to quantify the relative levels of HO-1 and NQO-1.

Nuclear and Cytosolic Protein Extraction. Nuclear and cytosolic proteins were fractioned using a Nuclear/Cytosol Fractionation Kit according to the methods provided by the instructions of the manufacturer (Biovision). Briefly, cells were collected and centrifuged at 600*g* for 5 min at 4 °C. The cell lysates were added to cytosol extraction buffer A containing dithiothreitol (DTT) and protease inhibitor (PI) and then vortexed vigorously for 15 s. After incubation for 10 min on ice, cells were added to Cytosol Extraction Buffer-B containing DTT and PI and then incubated on ice for 1 min followed by centrifugation at 16000*g* for 5 min. The supernatant cytoplasmic fraction was transferred to new tubes and stored at −80 °C until used, and then the lysates were resuspended in Nuclear Extraction Buffer followed by vigorous vortexing for 10 min four times. After centrifugation, the supernatants (nuclear fractions) were collected and stored at −80 °C until used.

Western Blotting. Protein expression of Nrf2 (nuclear), Keap1 (cytosol), HO-1, NQO-1, and MAPK families in HepG2 cells was determined using Western blotting. Briefly, cellular total proteins were extracted using RIPA buffer containing protease inhibitors and were centrifuged for 30 min at 4 °C. The proteins (60 μg) from the supernatant were resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% nonfat milk for 1 h and then was incubated with different primary antibodies. The membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h followed by visualization using an ECL Chemiluminescent Detection Kit (Amersham Co., Bucks, U.K.). The relative density of the protein expression was quantitated by densitometry (Matrox Inspector 2.1 software).

Electrophoretic Mobility Shift Assay (EMSA). The Nrf2 and ARE binding activities in nuclear fraction were determined by EMSA using a Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL), as described previously.[50] The double-stranded oligonucleotides were based on sequences of biotin-labeled double-stranded Nrf2 (5′-CTACGATTTCTGCTTAGTCATTGTCTTCC-3′) in the ARE promoter. Briefly, 5 μg of nuclear extract was incubated with 5× binding buffer, poly d(I−C) (1 μg/μL), ARE probe (10 ng/μL), biotin-labeled double-stranded Nrf2 oligonucleotides (Rockford, IL), and distilled water for 30 min at room temperature. Competition was performed using the unlabeled Nrf2 oligonucleotides. Specific binding was confirmed by using a 200-fold excess of unlabeled probe as a specific competitor. Protein−DNA complexes were separated by using a 6% acrylamide gel electrophoresis and then transferred onto an immobile membrane (nylon; Millipore, Bedford, MA). The membrane was incubated with the 1× blocking buffer for 15 min at room temperature and immersed in blocking buffer that contained

streptavidin–horseradish peroxidase. The membrane was washed three times at room temperature with 20 mL of 1× wash buffer. Gel shifts were visualized with enhanced chemiluminescence (ECL) Western blotting reagents. The relative binding activities of Nrf2 and ARE were quantitated by Matrox Inspector 2.1 software.

Transient Transfection and Luciferase Reporter Gene Assay. To construct p3xARE/Luc, tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site, 5′-TGACTCAGCA-3′[51] were introduced into the restriction sites of pGL3 promoter plasmid (Promega). Transfection of p3xARE/Luc-pGL3 into HepG2 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).

Transient Transfection and RNA Silencing against Nrf2. The plasmid containing siRNA against Nrf2 (siNrf2: the coding region nucleotides 1903–1921, 5′-GTAAGAAGCCAGATGTTAA-3′, in the Nrf2 cDNA) and the DharmaFECT transfection reagent were purchased from Thermo Scientific Dharmacon. The Nrf2 siRNA duplex with the following sense and antisense sequences was used: 5′-GUAAGAAGCCAGAUGUUAAdUdU-3′ (sense) and 3′-dUdU-CAUUCUUCGGUCUACAATT-5′ (antisense). All of the siRNA duplexes were synthesized by Dharmacon Research (Lafayette, CO). Briefly, siNrf2 stock solutions (20 μM) were diluted with DEPC water to form 5 μM solutions at room temperature. The DharmaFECT transfection reagent was incubated with 5 μM siNrf2 for 20 min and then added to the culture medium at a final concentration of 70 nM. Cells were transfected with siNrf2 (70 nM) for 60 h using DharmaFECT transfection reagent according to the manufacturer's recommendations.

Determination of Chymotrypsin-like Activity. The chymotrypsin-like activity of the proteasome in cell lysates was measured by using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr linked to the fluorometric reporter aminomethyl coumarin (AMC), as described previously.[52] Briefly, cell lysates were prepared as described under Western Blotting and were incubated for 20 min in a reaction buffer containing an ATP regenerating system (50 mM Tris-HCl, pH 8.2, 18 mM KCl, 3 mM $Mg(CH_3COO)_2$, 3 mM $MgCl_2$, 1.1 mM DTT, 6 mM ATP, 5 mM phosphocreatine, 0.2 U phosphocreatinkinase) and 0.2 mM Suc-Leu-Leu-Val-Tyr AMC. AMC hydrolysis was measured using a spectroflorometer with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Enzymatic activity was normalized to protein concentration and expressed as percentage activity of control lysates.

Determination of Intracellular ROS Levels. Flow cytometry and spectrofluorometry are generally used to measure the intracellular ROS levels. As HepG2 cells form aggregates in cultured media, flow cytometry is not suitable for the determination of ROS levels in this study. Therefore, we detected the intracellular ROS levels using the redox-sensitive fluorescent probe 2′,7′-dichlorofluorescenin diacetate (DCFDA) by spectrofluorometry. Cells (10^5 cells/mL) were seeded onto a 6 cm dish and incubated with different concentrations of apo-8′-lycopenal (1–10 μM) for 3, 6, 8, 12, and 24 h. After treatment, cells were incubated with 10 μM DCFDA for 1 h in the dark, and then cells were washed with PBS and scraped into ddH_2O followed by sonication. The fluorescence of H_2-DCFDA stained cells was measured using a spectrofluorometer (excitation wavelength, 485 nm; emission wavelength, 520 nm). The percentages of ROS levels were estimated by comparison with the control group.

Statistical Analysis. Values, which are expressed as the mean ± SD, were analyzed using one-way ANOVA followed by least significant difference (LSD) test for comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a P value of <0.05 is considered to be statistically significant.

RESULTS

Time Course Effects of Apo-8′-lycopenal or Lycopene on Nuclear Nrf2 Accumulation in HepG2 Cells. We first investigated whether 10 μM apo-8′-lycopenal increases nuclear Nrf2 accumulation in HepG2 cells during 0–24 h of incubation in comparison with 10 μM lycopene. Apo-8′-lycopenal significantly increased nuclear Nrf2 accumulation during 24 h of incubation, and the effect was strongest at 6 h of incubation by 2.8-fold (P < 0.01) (Figure 1). As expected, lycopene

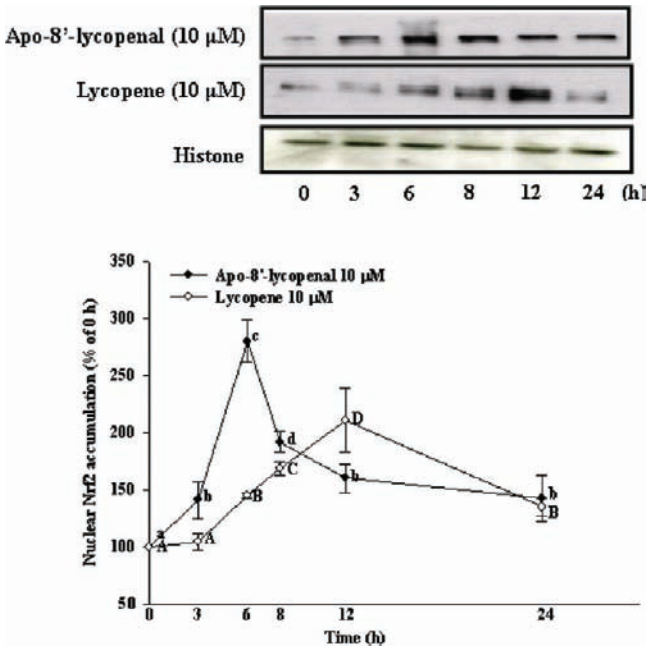

Figure 1. Time course effect of apo-8′-lycopenal or lycopene on nuclear Nrf2 accumulation in HepG2 cells (upper panel, Western blot of nuclear Nrf2 accumulation; lower panel, densitometric analysis). HepG2 cells were incubated with apo-8′-lycopenal (10 μM) or lycopene (10 μM) for 0–24 h. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly (P < 0.05).

significantly increased the nuclear Nrf2 accumulation, and the effect was strongest at 12 h of incubation by 2.1-fold (P < 0.01) (Figure 1).

Concentration Effects of Apo-8′-lycopenal on Nuclear Nrf2 Accumulation and Nrf2-ARE Binding Activity. On the basis of the time course results of nuclear Nrf2 accumulation, we chose the most effective time (6 h of incubation) to determine the concentration effect of apo-8′-lycopenal (1–10 μM) on nuclear Nrf2 accumulation and Nrf2-ARE binding activity using Western blotting, ARE-luciferase assay, and EMSA. We found that apo-8′-lycopenal significantly and concentration-dependently increased nuclear Nrf2 accumulation by 2.4-fold (P < 0.01) (Figure 2A), ARE-luciferase activity by 6.8-fold (P < 0.01) (Figure 2B), and Nrf2-ARE binding activity by 4.4-fold (P < 0.01) (Figure 2C) in HepG2 cells, as compared with the control group.

Effects of Apo-8′-lycopenal on Cytosolic Keap1 Protein Expression and Proteasome Activity. We determined the time course effect of apo-8′-lycopenal on Keap1 protein expression in HepG2 cells during 24 h of incubation. Results reveal that apo-8′-lycopenal significantly and transiently decreased cytosolic Keap1 protein expression during 8 h of incubation, but the effect disappeared at 12 and 24 h of incubation. The strongest inhibition occurred at 6 h of incubation, with an inhibition of 35% (P < 0.05) at 10 μM apo-8′-lycopenal (Figure 2D). In addition, incubation of

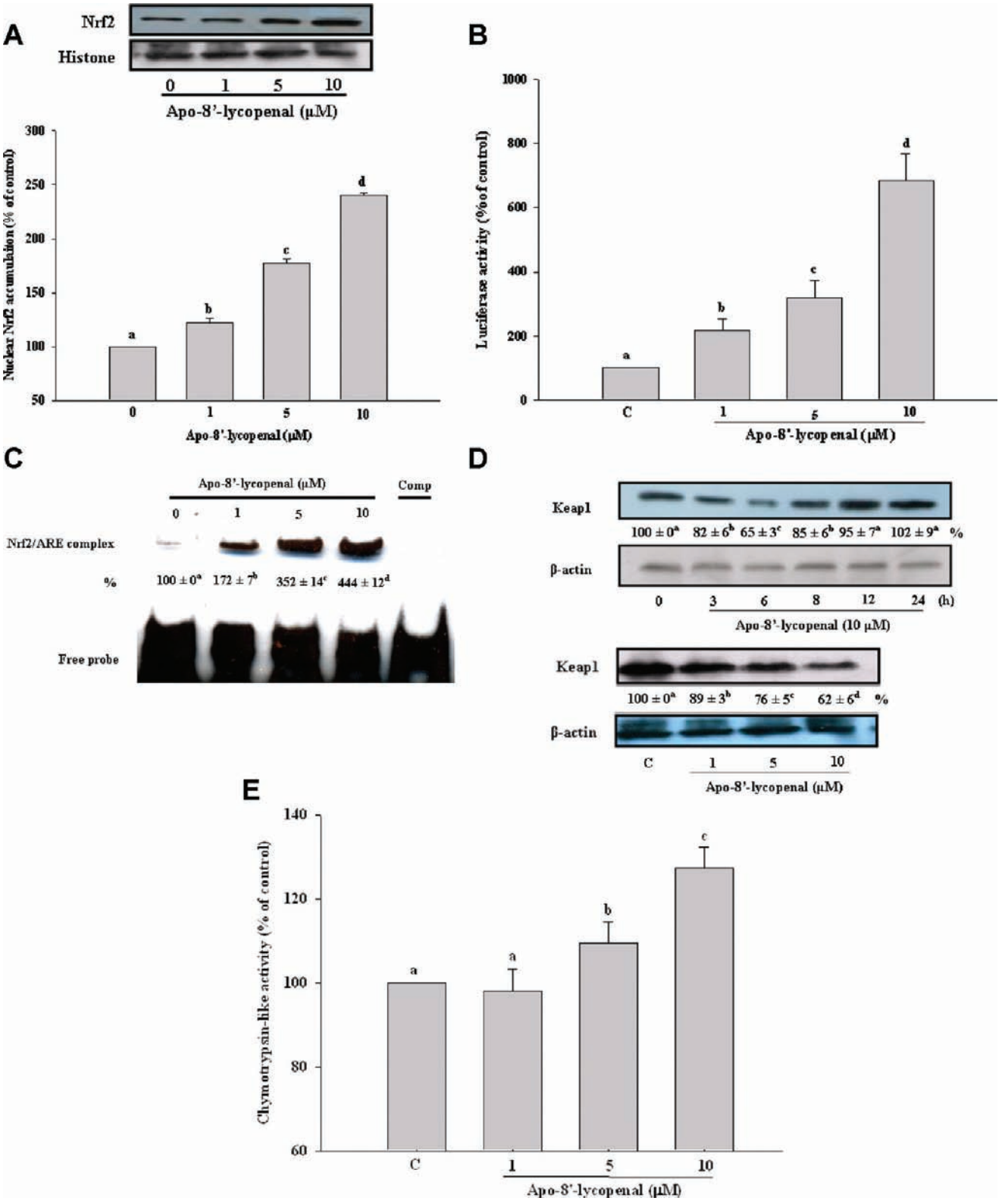

Figure 2. Effects of apo-8′-lycopenal on nuclear Nrf2 accumulation, ARE luciferase activity, Nrf2-ARE binding activity, cytosolic Keap1 protein expression, and chymotrypsin-like activity at 6 h of incubation in HepG2 cells. HepG2 cells were treated with apo-8′-lycopenal (0−10 μM) for 6 h: (A) Western blot of nuclear Nrf2 accumulation (upper panel) and densitometric analysis (lower panel); (B) ARE luciferase activity; (C) EMSA for Nrf2-ARE binding activity ("Comp" represents nuclear extracts treated with unlabeled oligonucleotide to confirm the specificity of binding); (D) Western blot for cytosolic Keap1 protein expression (upper panel, time course effects of apo-8′-lycopenal; lower panel, concentration effects of apo-8′-lycopenal); (E) chymotrypsin-like activity. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

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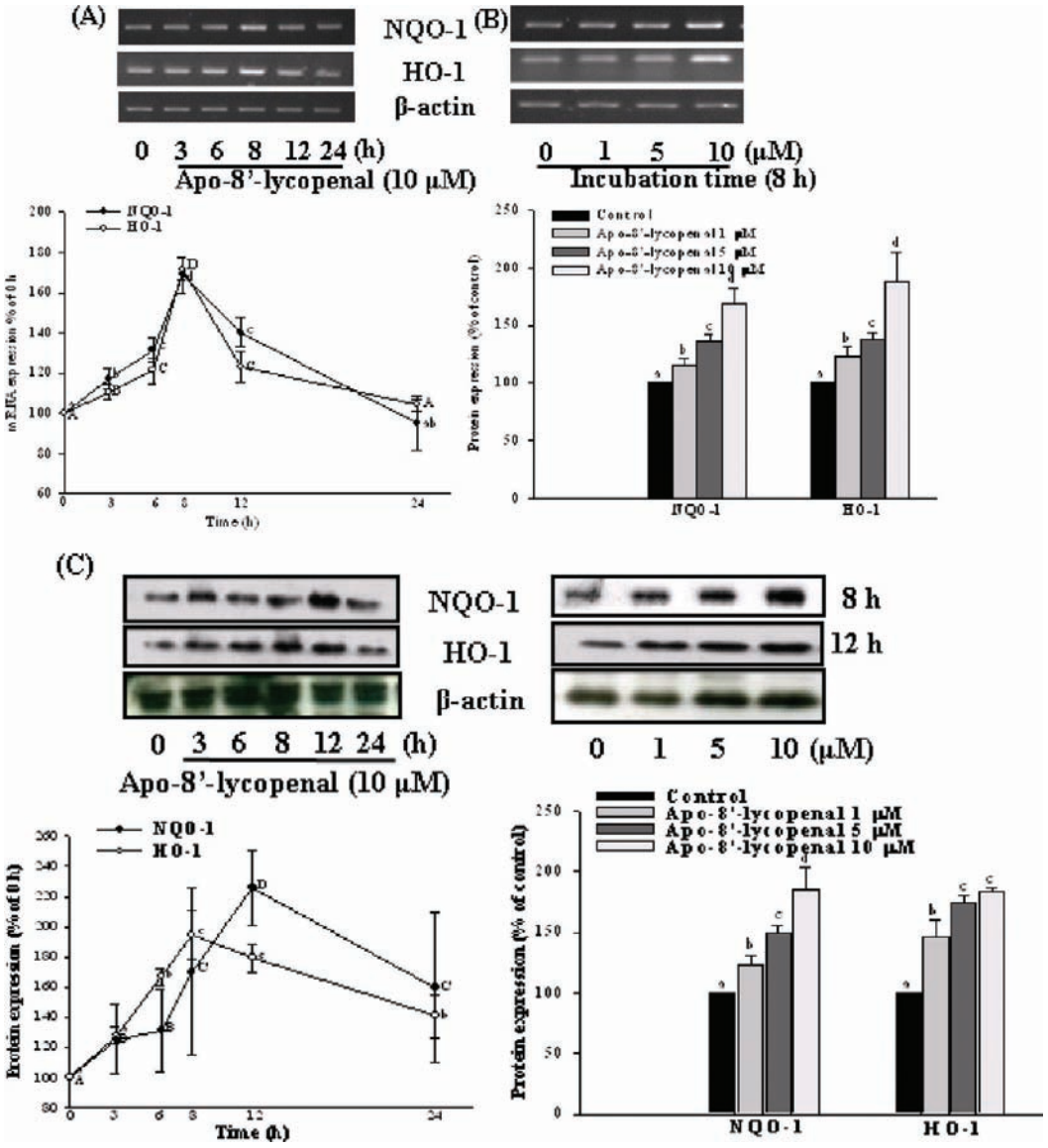

Figure 3. Time course and concentration-dependent effects of apo-8′-lycopenal on both mRNA and protein expression of NQO-1 and HO-1 in HepG2 cells. In time course experiments, HepG2 cells were treated with apo-8′-lycopenal (10 μM) for 0−24 h. In concentration-dependent experiments, HepG2 cells were incubated with apo-8′-lycopenal (0−10 μM) for the indicated times: (A) mRNA expression of NQO-1 and HO-1 (upper panel) and densitometric analysis (lower panel); (B) mRNA expression of NQO-1 and HO-1 (upper panel) and densitometric analysis (lower panel); (C) protein expression of NQO-1 and HO-1 (upper panel) and densitometric analysis (lower panel); (D) protein expression of NQO-1 and HO-1 (upper panel) and densitometric analysis (lower panel). Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

HepG2 cells with apo-8′-lycopenal (0−10 μM) for 6 h significantly decreased cytosolic Keap1 protein expression in a concentration-dependent manner with an inhibition of 38% ($P < 0.05$) at 10 μM apo-8′-lycopenal (Figure 2D).

We further determined the effect of apo-8′-lycopenal on chymotrypsin-like activity, an indicator of proteasome activity, in HepG2 cells. Treatment of HepG2 cells with apo-8′-lycopenal (0−10 μM) for 6 h resulted in a significant increase of chymotrypsin-like activity in a concentration-dependent manner with a promotion of 27% ($P < 0.05$) at 10 μM apo-8′-lycopenal (Figure 2E).

Time Course and Concentration Effects of Apo-8′-lycopenal on mRNA and Protein Expression of NQO-1 and HO-1. Treatment of HepG2 cells with apo-8′-lycopenal (10 μM) for 0−24 h significantly increased mRNA expression of NQO-1 and HO-1, and these effects were strongest at 8 h of incubation with a promotion of 68% ($P < 0.05$) and 71% ($P < 0.05$), respectively, but these effects disappeared at 24 h of incubation (Figure 3A). It has been shown that the half-life of HO-1 is about 3 h under normal conditions in several cell lines.[53−55] Therefore, it is possible that the inductive effect of apo-8′-lycopenal on HO-1 and NQO-1 expression disappeared at 24 h, possibly because of the short half-life of HO-1 and NQO-1. On the basis of the time course findings, we chose the most effective time (8 h of incubation) to determine the concentration effect of apo-8′-lycopenal on mRNA expression of NQO-1 and HO-1. We found that apo-8′-lycopenal significantly increased mRNA expression of NQO-1 and HO-1 in a concentration-dependent manner with a promotion of

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69% ($P < 0.05$) and 88% ($P < 0.05$), respectively, at 10 μM apo-8′-lycopenal treatment (Figure 3B).

Incubation of HepG2 cells with 10 μM apo-8′-lycopenal for 0−24 h resulted in significant increases in the protein expression of NQO-1 and HO-1, and these effects were strongest at 12 and 8 h of incubation (by 125%, $P < 0.05$; and 94%, $P < 0.05$, respectively), respectively (Figure 3C). We then chose the most effective time (12 h for NQO-1 and 8 h for HO-1 of incubation) to determine the concentration effect of apo-8′-lycopenal on protein expression of NQO-1 and HO-1. We found that apo-8′-lycopenal significantly and concentration-dependently increased the protein expression of NQO-1 and HO-1 by 85% ($P < 0.05$) and 83% ($P < 0.05$), respectively (Figure 3D). In addition, we found that apo-8′-lycopenal (1−10 μM) did not affect cell proliferation at 12 h of incubation, and there was only a slight inhibition of proliferation (~14%) at 10 μM apo-8′-lycopenal incubated for 24 h (Figure 4).

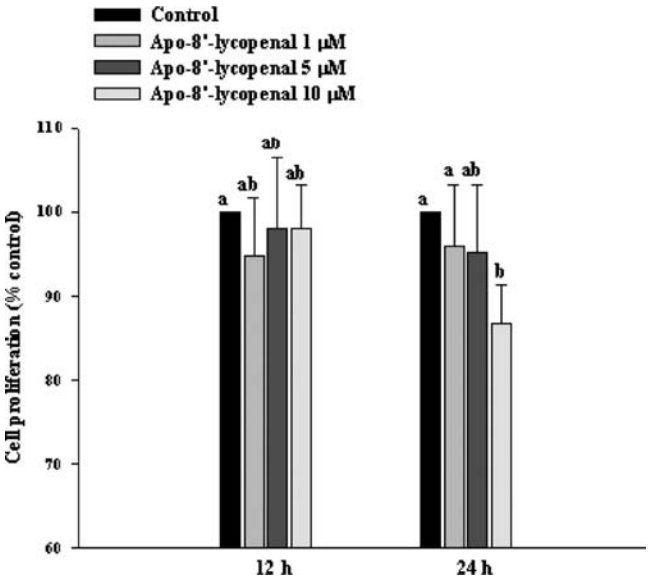

Figure 4. Effects of apo-8′-lycopenal on cell proliferation in HepG2 cells. HepG2 cells were treated with apo-8′-lycopenal (0−10 μM) for 12 and 24 h. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

Effects of Apo-8′-lycopenal on Phosphorylation and Protein Expression of MAPK Family. HepG2 cells were incubated with apo-8′-lycopenal (10 μM) for 0−6 h to determine the phosphorylation and protein expression of MAPK members, including ERK, JNK, and p38. Results revealed that apo-8′-lycopenal significantly increased the phosphorylation of ERK and p38 at 2 h of incubation without affecting the phosphorylation of JNK and the protein expression of ERK, JNK, and p38 during 6 h of incubation (Figure 5).

We further used the specific inhibitors for ERK (PD98059) and p38 (SB203580) to confirm the role of ERK and p38 in apo-8′-lycopenal-induced Nrf2-mediated NQO-1 and HO-1 in HepG2 cells. HepG2 cells were pretreated with PD98059 (40 μM) or SB203580 (20 μM) for 1 h and then were incubated with apo-8′-lycopenal (10 μM) for additional indicated times (6 h for Nrf2, 8 h for HO-1, 12 h for NQO-1). The effectiveness of these inhibitors was confirmed by the use of ERK1/2 and p38 inhibitors PD98059 and SB203580, both of which

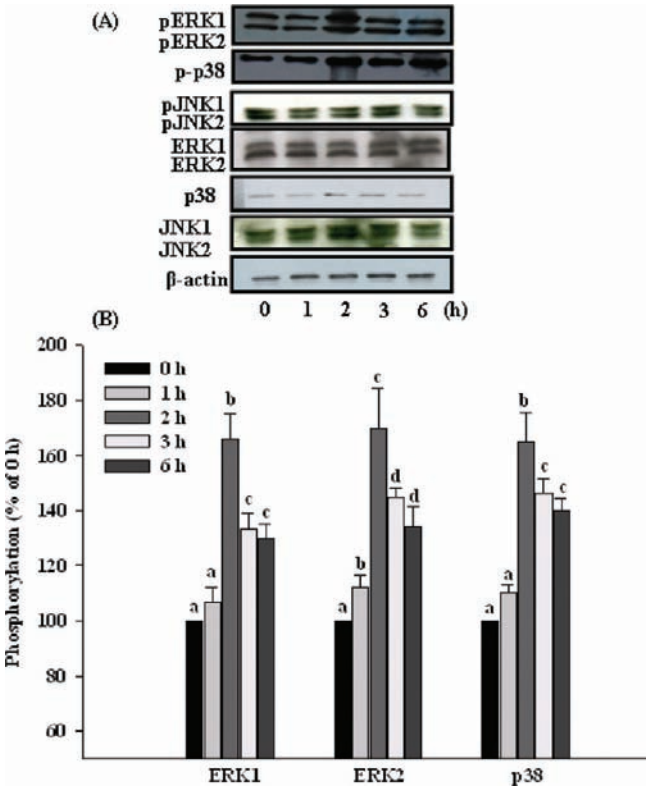

Figure 5. Effects of apo-8′-lycopenal on phosphorylation and protein expression of ERK, p38, and JNK in HepG2 cells. HepG2 cells were treated with apo-8′-lycopenal (10 μM) for 0−6 h: (A) Western blot for phosphorylation and protein expression of ERK, p38, and JNK; (B) densitometric analysis of phosphorylation of ERK and p38. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

abolished apo-8′-lycopenal-induced phosphorylation of ERK1/2 and p38 (Figure 6A). As expected, apo-8′-lycopenal increased nuclear Nrf2 accumulation and protein expression of HO-1 and NQO-1, whereas inhibition of ERK1/2 and p38 pathways strongly decreased these effects of apo-8′-lycopenal. (Figure 6B,C). These results indicate that ERK and p38 are involved in apo-8′-lycopenal-induced activation of Nrf2-mediated expression of HO-1 and NQO-1 in HepG2 cells.

Effects of Silencing Nrf2 on Apo-8′-lycopenal-Induced HO-1 and NQO-1 Expression. We further confirmed the role of Nrf2 on apo-8′-lycopenal-induced HO-1 and NQO-1 expression using small interfering RNA inhibition of Nrf2. HepG2 cells were pretreated with Nrf2 siRNA for 60 h and then incubated with apo-8′-lycopenal (10 μM) for indicated times (6 h for Nrf2, 8 h for HO-1, 12 h for NQO-1). The effectiveness of RNA silencing was confirmed by the use of siNrf2, which abolished the nuclear Nrf2 accumulation and protein expression of HO-1 and NQO-1 (Figure 7A). As expected, apo-8′-lycopenal increased nuclear Nrf2 accumulation and protein expression of HO-1 and NQO-1, whereas Nrf2 RNA silencing strongly decreased these effects of apo-8′-lycopenal (Figure 7B). Thus, these results indicate that apo-8′-lycopenal can induce phase II detoxifying/antioxidant enzymes by the activation of the Nrf2-ARE pathway.

Effects of Apo-8′-lycopenal on Intracellular ROS Levels. Incubation of HepG2 cells with apo-8′-lycopenal (0−10 μM) for 12 and 24 h exerted a significant and concentration-

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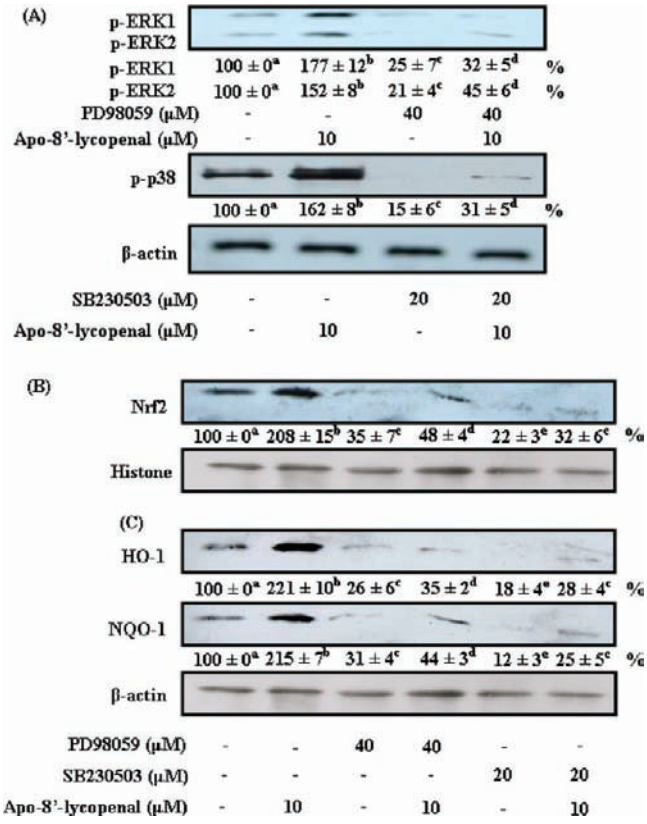

Figure 6. Effects of apo-8′-lycopenal alone or in combination with or without the specific inhibitor for ERK and p38 on nuclear Nrf2 accumulation and protein expression of HO-1 and NQO-1 in HepG2 cells. HepG2 cells were treated with specific inhibitor for ERK (PD98059, 40 μM) and p38 (SB230503, 20 μM) for 1 h followed by incubation with apo-8′-lycopenal (10 μM) for additional indicated times (6 h for Nrf2, 8 h for NQO-1, and 12 h for HO-1): (A) Western blot for nuclear Nrf2 accumulation; (B) Western blot for protein expression of HO-1 and NQO-1. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

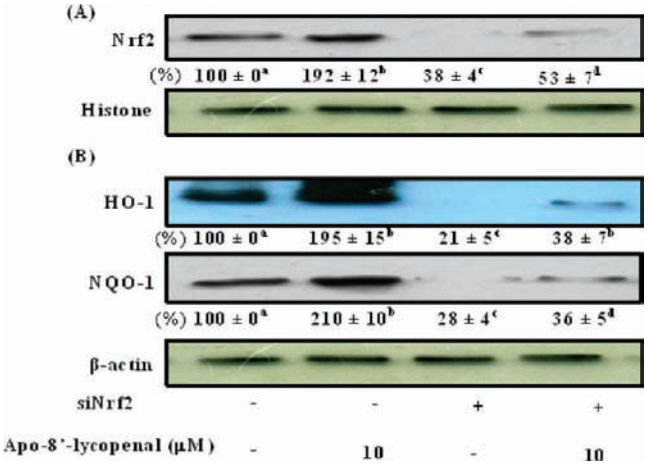

Figure 7. Effects of apo-8′-lycopenal alone or in combination with or without Nrf2 siRNA on nuclear Nrf2 accumulation and protein expression of HO-1 and NQO-1 in HepG2 cells. HepG2 cells were treated with Nrf2 siRNA for 60 h followed by incubation with apo-8′-lycopenal (10 μM) for additional indicated times (6 h for Nrf2, 8 h for NQO-1, and 12 h for HO-1): (A) Western blot for nuclear Nrf2 accumulation; (B) Western blot for protein expression of HO-1 and NQO-1. Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

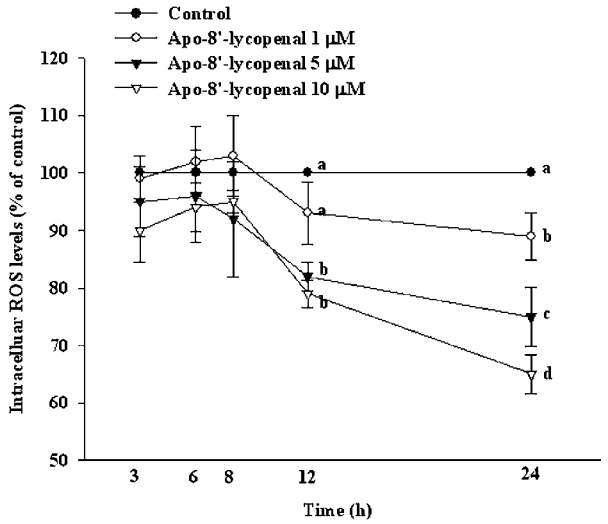

Figure 8. Effects of apo-8′-lycopenal on intracellular ROS levels in HepG2 cells. HepG2 cells were treated with apo-8′-lycopenal (0−10 μM) for up to 24 h. After the treatment, HepG2 cells were incubated with 2′,7′-dichlorofluorescenin diacetate (DCFDA) for an additional 1 h. Intracellular ROS levels were determined using a spectrofluorometer (excitation wavelength, 485 nm; emission wavelength, 520 nm). Data (mean ± SD) are from three or four separate experiments; means without a common letter differ significantly ($P < 0.05$).

dependent inhibition of intracellular levels of ROS (Figure 8). This inhibitory effect was stronger at 24 h than at 12 h of incubation, with an inhibition of 35% ($P < 0.05$) at 10 μM apo-8′-lycopenal for 24 h of incubation (Figure 8). In contrast, apo-8′-lycopenal did not affect the levels of intracellular ROS at incubation times shorter than 12 h (Figure 8).

DISCUSSION

The main question addressed by this study was whether apo-8′-lycopenal, one of the major enzymatic metabolites of lycopene in the rat liver, in lycopene-containing food and in human plasma, induces phase II detoxifying/antioxidant enzymes through the Nrf2-ARE system. We found for the first time that apo-8′-lycopenal induced nuclear Nrf2 accumulation, Nrf2-ARE binding activity, and downstream phase II detoxifying/antioxidant enzyme expression including HO-1 and NQO-1, leading to reduced intracellular ROS levels in HepG2 cells. Another lycopene metabolite, apo-12′-lycopenoic acid, has previously been found to induce phase II detoxifying/antioxidant enzymes through the Nrf2-ARE transcriptional system.[41] Intriguingly, Linnewiel et al. have reported that hydrophilic oxidation products of lycopene, but not the intact lycopene, are the activator of the Nrf2-ARE transcription system in MCF-7 cells.[43] This is in contrast with our present finding that lycopene itself was able to significantly increase nuclear Nrf2 accumulation in HepG2 cells, although the activation time of Nrf2 accumulation by lycopene was somewhat slower than that by apo-8′-lycopenal (Figure 1). Our present results are in agreement with those reported by Ben-dor et al. that lycopene itself is able to activate the Nrf2-ARE system in MCF-7 and HepG2 cells.[37]

Many physiological and pathological processes are mediated by MAPK members, including ERK, p38, and JNK.[56] Here, we

demonstrated that the ERK/p38-Nrf2 pathway was involved in activation of HO-1 and NQO-1 expression by apo-8′-lycopenal. The involvement of ERK/p38-Nrf2 pathways in the activation of HO-1 and NQO-1 expression by apo-8′-lycopenal in HepG2 cells was confirmed by using the Nrf2-siRNA technique and the specific inhibitors of MAPK, both of which markedly prevented apo-8′-lycopenal-induced HO-1 and NQO-1 expression. However, our results do not exclude the participation of other upstream signaling molecules or other transcription factors in the activation of HO-1 and NQO-1 expression induced by apo-8′-lycopenal, because neither Nrf2-siRNA nor ERK/p38 specific inhibitors completely abolished apo-8′-lycopenal-induced nuclear Nrf2 accumulation and downstream HO-1 and NQO-1 expression. We did not determine the effect of apo-8′-lycopenal on the phosphorylation of ERK1/2 and p38 at 12 and 24 h of incubation because the activation of upstream kinases, such as MAPK family, is known to induce Nrf2 phosphorylation, which causes the dissociation of Nrf2 from its repressor Keap1 and subsequent translocation into nucleus.[5] Furthermore, the activation of protein kinases is known to be rapid and transient.[57,58]

Another possible mechanism by which apo-8′-lycopenal promotes the translocation of Nrf2 to the nuclei is the dissociation of Nrf2 from its intrinsic inhibitor Keap1 through modification of thiol groups in Keap1 protein. The modified Keap1 protein can then be degraded by the ubiquitin-proteasome pathway, which plays an important role in many cellular processes.[59] In eukaryotes, the proteasome is located in the nucleus and the cytosol and capable of degrading unneeded, misfolded, and damaged protein.[60] Several phytochemicals, such as zerumbone (a sesquiterpene isolated from tropical ginger),[61,62] chalcone (a kind of flavonoid),[63] and curcumin,[14,18] have been shown to activate the Nrf2-ARE system through the α,β-unsaturated carbonyl group that acts as Michael acceptor. For instance, zerumbone and curcumin increase nuclear Nrf2 accumulation; in contrast, its analogues, such as 8-hydroxy-α-humulene and tetrahydrocurcumin, which lack an α,β-unsaturated carbonyl group, fail to activate nuclear Nrf2 accumulation,[18,62] indicating that an α,β-unsaturated carbonyl moiety is critical for Nrf2 activation.[64] Similarly, apo-8′-lycopenal has an α,β-unsaturated carbonyl moiety between the C1 and C2 positions, and the highly reactive aldehyde group may directly modify the cysteine residues in Keap1, resulting in the release and translocation of the Nrf2 protein to the nuclei. Moreover, it has been shown that the ethanolic oxidation product of lycopene containing an electrophilic unsaturated ketone group can interact with Keap1 and trigger the Nrf2 system.[37] Herein, we demonstrated that apo-8′-lycopenal increased nuclear Nrf2 accumulation and Nrf2-ARE binding activity, whereas this lycopene metabolite decreased cytosolic Keap1 protein expression but increased proteasome activity. These results suggest that apo-8′-lycopenal is able to react with Keap1 protein and render it digestible by the proteasome.

Carotenoids have been shown to exert antioxidant effects both in vitro and in vivo.[65] In the present study, we found that apo-8′-lycopenal decreased intracellular ROS levels at the incubation time of 12 and 24 h in HepG2 cells, indicating that apo-8′-lycopenal functions as an antioxidant. Our time course experiments show that apo-8′-lycopenal did not affect intracellular levels of ROS at incubation times shorter than 12 h. The result suggests that the ability of apo-8′-lycopenal in decreasing levels of ROS is likely associated with activation of Nrf2-ARE-mediated phase II antioxidant/detoxifying enzymes. This result is also consistent with previous findings[41] that apo-10′-lycopenoic acid, another lycopene metabolite, decreases intracellular ROS through the Nrf2-ARE system. However, our results do not exclude the possibility that apo-8′-lycopenal may directly scavenge ROS because phytoene, the precursor of lycopene, has been shown to decrease intracellular ROS without activating the ARE.[37]

Although the present study has provided new knowledge of the molecular mechanisms for the chemopreventive effect of apo-8′-lycopenal, it should be noted that the concentrations of apo-8′-lycopenal (1−10 μM) or lycopene (10 μM) used in the present study are supraphysiological (for lycopene) and pharmacological or even toxicological (for apo-8′-lycopenal). In healthy populations or in rat livers, the plasma levels of apo-8′-lycopenal are in nanomolar or picomolar levels after the consumption of tomato juice for 8 weeks[45] or after the consumption of 8 mg/day lycopene for 30 days, respectively.[44] Similarly, the plasma levels of apo-10′-lycopenoic acid are around 17 nM after supplementation with apo-10′-lycopenoic acid (140 mg/kg/day in diet) for 14 weeks in A/J mice.[40] In addition, the plasma lycopene concentration in healthy humans is around 0.5 μM.[66] After the consumption of 18.4 mg/day lycopene from 240 g of tomato juice for 3 weeks, the plasma levels of lycopene increase from 428 to 960 nM.[67]

In summary, our current study demonstrates that the lycopene metabolite apo-8′-lycopenal can activate upstream signaling pathways, such as ERK1/2 and p38, followed by up-regulation of the expression of phase II detoxifying/antioxidant enzymes, including HO-1 and NQO-1, mediated by the Nrf2-ARE transcription system, leading to decreased levels of endogenous ROS in HepG2 cells. In addition, these results suggest that the chemopreventive effect of lycopene may be, at least in part, contributed by its metabolites.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ARE, antioxidant response element; DCFDA, 2′,7′-dichlorofluorescenin diacetate; EGCG, epigallocatechin gallate; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; NQO-1, NAD(P)H:quinine oxidoreductase 1; Nrf2, nuclear factor erythroid-derived 2-like.

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