Carotenoids activate the antioxidant response element transcription system

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Abstract

Epidemiologic studies have found an inverse association between consumption of tomato products and the risk of certain types of cancers. However, the mechanisms underlying this relationship are not completely understood. One mechanism that has been suggested is induction of phase II detoxification enzymes. Expression of phase II enzymes is regulated by the antioxidant response element (ARE) and the transcription factor Nrf2 (nuclear factor E_2-related factor 2). In this study, we determined the role of this transcription system in the induction of phase II enzymes by carotenoids. We found that in transiently transfected cancer cells, lycopene transactivated the expression of reporter genes fused with ARE sequences. Other carotenoids such as phytoene, phytofluene, β-carotene, and astaxanthin had a much smaller effect. An increase in protein as well as mRNA levels of the phase II enzymes NAD(P)H:quinone oxidoreductase and γ-glutamylcysteine synthetase was observed in nontransfected cells after carotenoid treatment. Ethanolic extract of lycopene containing unidentified hydrophilic derivatives of the carotenoid activated ARE with similar potency to lycopene. The potency of the carotenoids in ARE activation did not correlate with their effect on intracellular reactive oxygen species and reduced glutathione level, which may indicate that ARE activation is not solely related to their antioxidant activity. Nrf2, which is found predominantly in the cytoplasm of control cells, translocated to the nucleus after carotenoid treatment.

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Interestingly, part of the translocated Nrf2 colocalized with the promyelocytic leukemia protein in the promyelocytic leukemia nuclear bodies. The increase in phase II enzymes was abolished by a dominant-negative Nrf2, suggesting that carotenoid induction of these proteins depends on a functional Nrf2 and the ARE transcription system. [Mol Cancer Ther 2005;4(1):177–86]

Introduction

There is considerable epidemiologic evidence suggesting an association between the consumption of fruits and vegetables and reduced incidence of cancer (1, 2). In particular, carotenoids and other plant constituents have been implicated as cancer-preventive agents (3). β-Carotene has received the most attention because of its provitamin A activity and its prevalence in many foods. However, findings from intervention studies with β-carotene were disappointing (4, 5), and thus other carotenoids such as lycopene, the main tomato carotenoid, became the subject of more intensive investigation. A comprehensive analysis of the epidemiologic literature on the relation of tomato consumption and cancer prevention has been published by Giovannucci (6). He found that most of the reviewed studies reported an inverse association between tomato intake or blood lycopene level and the risk of various types of cancer. Giovannucci suggested that lycopene may contribute to these beneficial effects of tomato-containing foods but that the anticancer properties could also be explained by interactions among multiple components found in tomatoes such as phytoene, phytofluene, and β-carotene. Additional support for the anticancer effects of these and other carotenoids was found with diverse cancer cells *in vitro* (7–10). For example, we have shown that lycopene inhibits mammary, endometrial, lung, and leukemic cancer cell growth in a dose-dependent manner (IC_{50} ~2 μmol/L; refs. 7, 8, 11).

The biochemical processes involved in the chemoprotective effects of fruits and vegetables are not completely understood. In recent years, evidence has accumulated indicating that the beneficial action is due, at least in part, to induction of phase II detoxification enzymes (12). These enzymes detoxify many harmful substances by converting them to hydrophilic metabolites that can be excreted readily from the body (13). Phase II enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1) and γ-glutamylcysteine synthetase (GCS) are highly inducible in animals and humans (14), and a strong inverse relationship exists between their tissue levels and susceptibility to chemical carcinogenesis (15). The coordinated induction of phase II enzymes is mediated through *cis*-regulatory DNA sequences located in the promoter or enhancer region, which are known as antioxidant

responsive elements (ARE; ref. 13). The major ARE-activating transcription factor Nrf2 (nuclear factor E$_2$-related factor 2) plays a central role in the induction of antioxidant and detoxifying genes. Under basal conditions, Nrf2 is located in the cytoplasm and is bound to an inhibitory protein, Keap1. Upon challenge with inducing agents, it is released from Keap1 and translocates to the nucleus (16, 17). Several studies have shown that dietary antioxidants, such as 1,2-dithiole-3-thiones (18), phenolic flavonoids (19, 20), curcuminoids (21), and isothiocyanates (22), may function as anticancer agents by activating this transcription system. Carotenoids, however, have not been studied in this regard. Because several carotenoids have been found to induce phase II enzymes (23, 24), we hypothesized that the mechanism of this induction involves activation of the ARE transcription system.

In the current study, we examined the activation of ARE and its role in the induction of phase II enzymes by different tomato carotenoids (lycopene, phytoene, phytofluene, and β-carotene) as well as the xantophyl astaxanthin, which is found in algae. Our results indicate that carotenoids activate ARE and suggest that this action is not solely related to their antioxidant activity but may be mediated by hydrophilic derivatives of the carotenoids.

Materials and Methods

Materials

Crystalline lycopene purified from tomato extract (>97% pure when prepared) was a gift from LycoRed Natural Products Industries (Beer-Sheva, Israel). Purified preparations of either phytoene or phytofluene were not available for this study. We used a mixture of the two carotenoids, purified by LycoRed from tomato extract, containing about 70% of phytoene. We are referring to this mixture in the paper as ''phytoene''. Astaxanthin and β-carotene were purchased from Sigma Chemicals (Rehovot, Israel). Tetrahydrofuran, containing 0.025% butylated hydroxytoluene as an antioxidant, was purchased from Aldrich (Milwaukee, WI). DMEM, MEM-Eagle medium, FCS, charcoal stripped, delipidated FCS, and Ca^{2+}/Mg^{2+}-free PBS were from Biological Industries (Beth Haemek, Israel). DMSO, glutathione, 5,5-dithiobis-(2-nitrobenzoic acid), and *tert*-butylhydroquinone (tBHQ) were purchased from Sigma.

Carotenoid and tBHQ Solutions

Lycopene, β-carotene, astaxanthin, and phytoene were dissolved in tetrahydrofuran and solubilized in cell culture medium as described previously (8). tBHQ was dissolved in DMSO. The final concentrations of tetrahydrofuran, DMSO, and ethanol in the cell culture media were 0.5%, 0.1%, and 0.1%, respectively. The vehicles did not affect the parameters measured in the presented experiments. All procedures were done under reduced lighting.

Ethanolic Extract of Lycopene Preparation

To obtain the extract, 35 mg of the crystalline lycopene preparation were extracted with 20 mL of ethanol. The extract was evaporated under vacuum yielding 11.5 mg solid (about 33% of the original lycopene) which was then redissolved in 3 mL of ethanol. The resulting ethanolic extract did not contain any detectable amounts of lycopene [as verified by measuring the absorption spectrum of the extract at 250 to 600 nm using the V 530 UV\VIS spectrophotometer (Jasco, Easton)]. The solubility of the extracted compounds in ethanol suggests that the extract contains less hydrophobic oxidation products of lycopene obtained during prolonged storage (−70°C). This suggestion is supported by the fact that preliminary high-performance liquid chromatography separation of the extract resulted in multiple peaks that are more hydrophilic than lycopene and have absorption spectra below 472 nm, which is characteristic of lycopene. In some of these peaks, the ''tri-finger'' absorption pattern characteristic of many carotenoids and their derivatives was observed. The total concentration of the compounds in the ethanolic extract is presented as the equivalent of the starting amount of lycopene.

Reporter Constructs and Expression Vectors

ARE reporter constructs were kindly provided by Dr. J.J. Gipp (University of Wisconsin Medical School, Madison, WI; ref. 25). NQO1hARE-*tk-luc* and GCS$_h$ARE4-*tk-luc* contain sequences of the active response elements from the promoters of human NQO1 and GCS heavy subunit, respectively. GCS$_h$ARE4m-*tk-luc*, a non-active mutant form of the latter construct, contains a single base mutation in the relevant sequence.

Expression vectors for wild-type Nrf2, a dominant-negative mutant Nrf2M, lacking the transactivation domain residues 1 to 392 in the NH$_2$-terminal portion of the protein and the empty vector (pEF; ref. 26), were a gift from Dr. J. Alam (Louisiana State University Medical Center, New Orleans, LA).

Cell Culture

MCF-7, human mammary cancer cells and HepG$_2$, human hepatocellular carcinoma cells were purchased from American Type Culture Collection (Rockwell, MD). MCF-7 cells were grown in DMEM containing penicillin (100 units/mL), streptomycin (0.1 mg/mL), nystatin (12.5 μg/mL), 0.6 μg/mL insulin, and 10% FCS. HepG$_2$ cells were grown in MEM-Eagle medium containing the antibiotics and 10% FCS.

Transient Transfection and Reporter Gene Assay

Cells were transfected using TFx-50 reagent (Promega, Madison, WI) with ∼0.07 μg of DNA containing 0.05 μg of reporter plasmid and 0.02 μg of *Renilla* luciferase expression vector (P-RL-null vector, Promega) as an internal standard. For this purpose, cells were seeded in 24-well plates (100,000 cells per well). After 1 day, cells were rinsed twice with the appropriate culture medium without serum, followed by the addition of 0.2 mL of medium containing DNA and TFx-50 reagent at a charge ratio of 1:3. The cells were then incubated for 30 minutes at 37°C in 95% air/5% CO$_2$. Four hundred microliters of medium containing 3% charcoal-stripped delipidated FCS were added and incubation continued for 8 hours. This was then replaced by medium supplemented with 3% delipidated FCS and the test

compounds and cells were incubated for another 16 hours. Cell extracts were prepared for luciferase reporter assay (Dual Luciferase Reporter Assay System, Promega) according to the manufacturer's instructions.

Stable Transfections

HepG$_2$ cells (2×10^6 in 100 mm plates) were transfected using TFx-50 reagent as described above with 5.5 μg of the expression plasmid DNA. Cells were incubated in medium containing 10% FCS for 2 days and then trypsinized and replated at 10,000, 20,000, and 100,000 cells per 100 mm plate in medium containing 750 mg/mL G418 (Calbiochem, Germany). Transfectants were selected after a 2-week period in the presence of G418 either as separate clones or as a mixture of 40 to 50 clones.

Real-time PCR

Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was prepared as previously described (27). NQO1 and GCS mRNA were determined by quantitative real-time PCR and the results were normalized according to corresponding values of glyceraldehyde-3-phosphate dehydrogenase mRNA. The following primers were used: NQO1 sense, 5'-CAACCACGAGCCCAGCCAAT A-3'; NQO1 antisense, 5'-TTCAAAGCCGCTGCAGCAG-3'; GCS sense, 5'-ACGAGGCTGAGTGTCCGTCT-3'; GCS antisense, 5'-TGGCGCTTGGTTTCCTC-3'; glyceraldehyde-3-phosphate dehydrogenase sense, 5'-GTTCGACAGTCAGCCGCATC-3'; glyceraldehyde-3-phosphate dehydrogenase antisense, 5'-CGCCCAATACGACCAAATCC-3'.

cDNA samples (7 μL) were diluted 9-fold, mixed with the specific primers (0.2 mmol) and Thermo-Start master mix (ABgene, Surrey, United Kingdom). SYBR green I dye (Amresco, Cleveland) was then added to the reaction mixture. Reactions were carried out in the Rotor-Gene Real-Time PCR machine (Corbett-Research, Northlake, Australia). Standard cycling conditions for this instrument were, 15 minutes initial enzyme activation at 95°C, then 35 cycles as follows: 10 seconds at 95°C, 15 seconds at the annealing temperature (60°C for NQO1 and GCS primers, 58°C for glyceraldehyde-3-phosphate dehydrogenase primer) and 20 seconds at 72°C.

Western Blotting

Cells were lysed as described previously (28) with modifications. Cell monolayers were washed twice with ice-cold PBS and then scraped into ice-cold lysis buffer A [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mmol/L MgCl$_2$, 1 mmol EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 10 mmoL sodium pyrophosphate, 50 mmol NaF, and 0.2 mmol/L DTT]. The lysates were incubated for 10 minutes on ice, and the cellular debris were cleared by centrifugation ($20,000 \times g$, 10 minutes, 4°C). The protein content of the samples was determined by the Bradford method using a protein assay kit (Bio-Rad, Richmond. CA). Equal amounts of protein (30 μg) were separated by SDS-PAGE and then transferred to a nitrocellulose membrane.

Proteins were visualized using the SuperSignal West Pico chemiluminescence system (Pierce Chemical, Rockford, IL) after incubation overnight at 4°C with the following primary antibodies: NQO1 (c-19, sc-16464), GCS (GSH1 N-20, sc-15085), Nrf2 (c-20, sc-722) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein abundance was quantitated by densitometric analysis using the ImageMaster VDS-CL imaging system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunostaining

HepG$_2$ cells were grown on cover slips, treated with tBHQ or carotenoids for 4 to 6 hours and processed for immunostaining as described previously (29). Briefly, the cells were washed with PBS and fixed in paraformaldehyde/lysine/periodate solution at room temperature for 10 minutes and then in 100% cold acetone for 10 minutes on ice. Then cells were washed with PBS and incubated with 6% goat serum for 1 hour at room temperature. For Nrf2 immunostaining, cells were first treated with an affinity-purified rabbit polyclonal anti-Nrf2 antibody (1000-fold dilution, sc-722; Santa Cruz Biotechnology) for 16 hours at 4°C, and then with 488 Alexa Fluor goat anti-rabbit IgG (A-11034; Molecular Probes, OR), for 1 hour at room temperature. For promyelocytic leukemia (PML) protein immunostaining, cells were incubated with mouse monoclonal anti-human PML IgG$_1$ antibody (40-fold dilution, sc-966; Santa Cruz Biotechnology) for 2 hours at 4°C and then with 568 Alexa Fluor goat anti-mouse IgG for 1 hour at room temperature. For DNA staining, cells were incubated for 5 minutes with 4',6-diamidino-2-phenyl-indole (Sigma). The cells were then examined by a fluorescence microscope (Axiovert 200M, Zeiss, Germany), equipped with the appropriate filter sets and a cooled CCD camera (Sensicam, PCO, Germany). Images were acquired and processed using MetaMorph data acquisition software (Universal Imaging).

Reduced Glutathione Determination

HepG$_2$ cells were seeded at 2×10^6 cells/100 mm plate. After 1 day, the medium was replaced with medium supplemented with 3% delipidated FCS, and cells were incubated for 24 hours in the presence of test compounds. At the end of the incubation period, cells were washed thrice with PBS, scraped and sonicated twice for 20 seconds and then centrifuged at $15,000 \times g$ for 10 minutes. The supernatant was mixed (1:1, v/v) with 1.7% metaphosphoric acid followed by centrifugation at $5,000 \times g$ for 10 minutes. Reduced glutathione (GSH) content in the supernatants was measured colorimetrically at 412 nm by the 5,5-dithiobis-(2-nitrobenzoic acid) reduction assay as described previously (30).

Measurement of Intracellular Reactive Oxygen Species by Flow Cytometry

Intracellular peroxide levels were determined using the oxidation-sensitive fluorescent probe 5,6-carboxyl-2',7'-dichlorofluorescein (31, 32). Intracellular peroxides oxidize this probe to a highly fluorescent compound, dichlorofluorescein. Cells were plated in 96-well microtiter plates

at a density of 8×10^5 cells per well (MCF-7 cells) or 6×10^5 cells per well (HepG$_2$ cells). After 1 day, the medium was changed to one containing the solubilized carotenoids or tBHQ and incubation continued for the indicated time. The medium was gently removed, and wells were rinsed with 150 μL RPMI containing 0.5% FCS serum and no phenol red or L-glutamine (33). The cells were incubated with the same medium containing 20 μmol/L 5,6-carboxyl-2′,7′-dichloro-fluorescein-diacetate (Sigma) for 30 minutes at 37°C. The medium was removed and 80 μL of PBS were added to each well. Fluorescence was measured (excitation, 485 nm; emission, 535 nm) using a Spectrafluor plus multifunctional plate reader (Tecan, Research Triangle Park, NC). To normalize data, cell number was measured after the fluorescence readings using the sulforhodamine B assay as described previously (34). Sulforhodamine B absorbance was measured at 492 nm in a VERSAmax microplate spectrophotometer (Molecular Devices, Menlo Park, CA).

Statistical Analysis

All experiments were repeated at least thrice. The significance of the differences between the means of the various subgroups versus corresponding controls in all figures (except Fig. 3) was assessed by two-tailed Student's *t* test using the Microsoft EXCEL program. In Fig. 3, statistically significant differences among the multiple groups were tested by one-way ANOVA followed by Newman-Keuls multiple comparison test using the Graph-Pad Prizm 3.0 program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Carotenoids Transactivate ARE Sequences of GCS and NQO1 Genes

To determine whether carotenoids are capable of activating ARE, we transiently transfected MCF-7 mammary cancer cells with a luciferase reporter vector containing ARE sequence derived from the promoter of the human GCS (GCS$_h$ARE4-*tk-luc*). tBHQ, a synthetic antioxidant which is a widely used ARE inducer (29, 35), served as the positive control. tBHQ transactivated this reporter gene up to 2- to 3-fold in a dose-dependent manner (0.3–30 μmol/L; Fig. 1A). Lycopene (6 μmol/L) was even more potent, reaching a 3- to 4-fold activation (Fig 1B). Similar results were obtained in GCS$_h$ARE4-*tk-luc*–transfected HepG$_2$ cells (data not shown). As expected, an inactive mutated construct designed with a point mutation within the ARE sequence (GCS$_h$ARE4m-*tk-luc*; ref. 25) was unable to support tBHQ or lycopene induction of luciferase expression (data not shown). In addition, cells transfected with the luciferase reporter vector, pT81, lacking the GCS$_h$ARE4 sequence, did not exhibit significant luciferase activity (data not shown).

We then compared the ability of different carotenoids to transactivate ARE in HepG$_2$ and MCF-7 cells, transiently transfected with two reporter genes GCS$_h$ARE4-*tk-luc* and a NQO1 reporter gene (NQO1hARE-*tk-luc*). As shown in Fig. 1C, lycopene induced a 3- to 4-fold increase in luciferase activity with both reporter constructs in HepG$_2$

cells. Similar results were obtained in MCF-7 cells (data not shown). The transactivation was more pronounced for lycopene, whereas astaxanthin, β-carotene, and phytoene exhibited a much lower effect which was statistically significant only for phytoene and astaxanthin with the GCS$_h$ARE4-*tk-luc* but not with NQO1hARE-*tk-luc* (Fig. 1C).

Carotenoids Increase NQO1 and GCS mRNA and Protein Levels

We determined whether carotenoids would also induce the ARE-driven phase II enzymes in intact cells, under more physiologic conditions. The effects of carotenoids and tBHQ on mRNA (Fig. 2A) and protein (Fig. 2B and C) levels of endogenous NQO1 and GCS were examined in non-transfected MCF-7 and HepG$_2$ cells using real-time PCR and Western blotting, respectively. We initially analyzed the kinetics of mRNA induction and found that maximal increase by tBHQ and lycopene was attained at 24 hours for NQO1 and at 6 hours for GCS. Then the effect of additional carotenoids on mRNA level was examined according to these time points. Lycopene and β-carotene induced a >2-fold increase in the level of mRNA of both genes. Phytoene and astaxanthin caused a slight increase which was statistically significant only for phytoene induction of
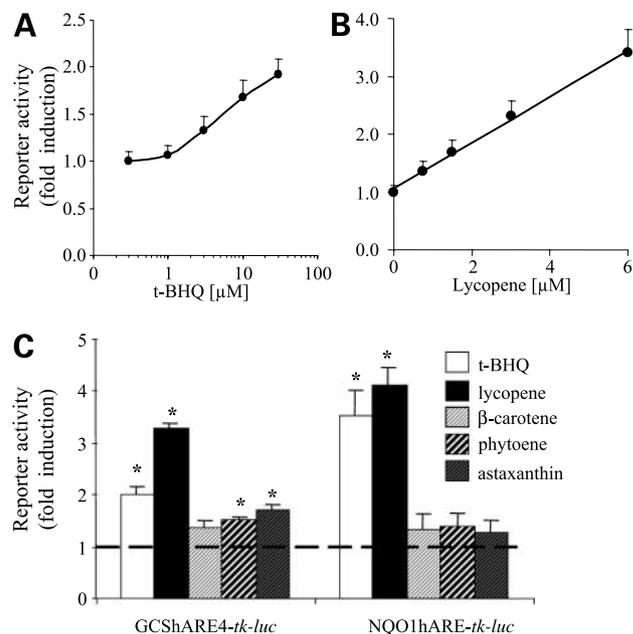
Figure 1. Transactivation of the ARE by carotenoids. MCF-7 cells were transiently transfected with GCS$_h$ARE4-*tk-luc* reporter plasmid as described under Materials and Methods, and treated for 16 h with the indicated concentrations of tBHQ (**A**) or lycopene (**B**). Transactivation of GCS$_h$ARE4-*tk-luc* or NQO1hARE-*tk-luc* reporter plasmids in HepG$_2$ cells by tBHQ and various carotenoids (**C**). Following transfection, cells were treated for 16 h with 0.5% tetrahydrofuran (control), tBHQ (30 μmol/L), lycopene (2–4 μmol/L), β-carotene (4–6 μmol/L), phytoene (4–6 μmol/L), or astaxanthin (7–10 μmol/L). Reporter activity is expressed as fold increase in luciferase activity, which was standardized to Renilla luciferase expression used as an internal control. Mean (*columns*) ± SE (*bars*) of three independent experiments, each done in triplicate. *, significantly different (*P* < 0.05) from the control (*dashed line*).
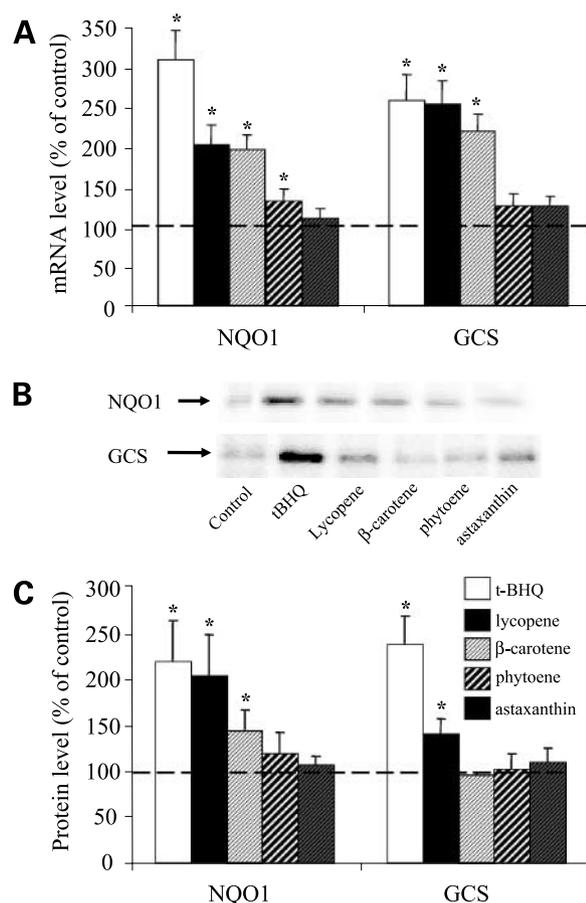
Figure 2. Effects of different carotenoids on mRNA (**A**) and protein (**B**) levels of NQO1 and GCS. HepG$_2$ cells (**A**) and MCF-7 cells (**B** and **C**) were treated with different carotenoids or tBHQ at the concentrations shown in Fig. 1C. **A,** total RNA was extracted from the cells and mRNA level was analyzed by real-time PCR as described under Materials and Methods. **B** and **C,** samples were analyzed by Western blotting for expression of NQO1 and GCS. **B,** representative blots. **C,** densitometric analysis of the Western blots. Results are expressed as the percentage of mRNA or protein level in control (tetrahydrofuran) cells. Mean (*columns*) ± SE (*bars*) of three to five experiments, each done in duplicate. *, significantly different ($P < 0.05$) from the control (*dashed line*).

NQO1. The increase in mRNA of NQO1 and GCS induced by tBHQ and lycopene was not accompanied by a change in the mRNA level of the ARE activating transcription factor Nrf2 (data not shown).

To determine the effect of carotenoids on phase II enzyme protein level, HepG$_2$ hepatoma cells were treated for 48 hours and analyzed for NQO1 and GCS expression by Western blotting (Fig. 2B and C). Densitometric analyses showed that lycopene caused a 50% and 150% increase in GCS and NQO1 protein level, respectively. Phytoene and astaxanthin exhibited lower potency in expression of these proteins. Similar results were obtained in MCF-7 cells (data not shown). The relative potency of lycopene, phytoene, and astaxanthin in the induction of GCS and NQO1 protein level (Fig. 2C) was similar to that obtained in the

transactivation studies (Fig. 1). In contrast, treatment with β-carotene, which showed no significant ARE transactivation, resulted in the induction of GCS and NQO1 mRNA and protein level which was similar to that caused by lycopene. This stimulation of expression by β-carotene may result from activation of other response elements in the promoters of these phase II enzymes.

Dominant-Negative Nrf2 Prevents Activation of ARE

To further explore the role of Nrf2 in phase II enzyme induction by carotenoids, we used HepG$_2$ cells ectopically expressing either the wild-type Nrf2 or its dominant-negative mutant, lacking the transactivation domain residues 1 to 392 in the NH$_2$-terminal portion of the protein (26). To determine the function of Nrf2 in the carotenoid ARE activation, cells were transiently cotransfected with one of the above expression vectors together with the NQO1hARE-*tk-luc* reporter plasmid. Control cells were transfected with the appropriate empty vector. As expected, tBHQ and lycopene stimulated reporter gene expression in cells transfected with the empty plasmid (Fig. 3A), similar to their effect in nontransfected cells (see Fig. 1). However, stimulation was totally inhibited in cells expressing the dominant-negative Nrf2 (Fig. 3A). These data indicate the efficiency of the dominant-negative Nrf2 to intervene with its endogenous intact counterpart. On the other hand, ectopic expression of the wild-type Nrf2 increased basal reporter gene activity but prevented most of the stimulation induced by tBHQ and lycopene (Fig. 3A). In a mixture of HepG$_2$ cells stably transfected with a wild-type Nrf2, an insignificant increase in the induction of NQO1 mRNA (Fig. 3B) and protein (Fig. 3C and D) by tBHQ and lycopene was observed. In order to examine the effect of the dominant-negative Nrf2 on NQO1 mRNA and protein induction, we stably transfected HepG$_2$ cells with the expression vector of this mutant. In the mixed population of cell clones expressing the dominant-negative Nrf2, the net accumulation of NQO1 mRNA in response to lycopene and tBHQ was reduced by about 50% and 70%, respectively (Fig. 3B). The negative effect of the mutant was even more pronounced when NQO1 protein level was examined. The induction of this enzyme by tBHQ and lycopene was totally abolished by the dominant-negative Nrf2 (Fig. 3C and D). Similar results were obtained with three isolated cell clones expressing the dominant-negative Nrf2 (results not shown). This further supports our hypothesis that the functional Nrf2 protein and the ARE transcription system mediate the induction of phase II enzymes by carotenoids.

Lycopene Oxidation Products Activate ARE

Various natural and synthetic chemicals have already been shown to activate ARE (see Introduction for details). However, none of these compounds are hydrophobic like the hydrocarbon carotenoids, lycopene, β-carotene, phytoene, and phytofluene tested here. Thus, it is possible that the effective compounds involved in ARE activation are oxidized derivatives of the carotenoids and not only their intact molecules. To test this hypothesis, we extracted the lycopene preparation with ethanol to separate the often
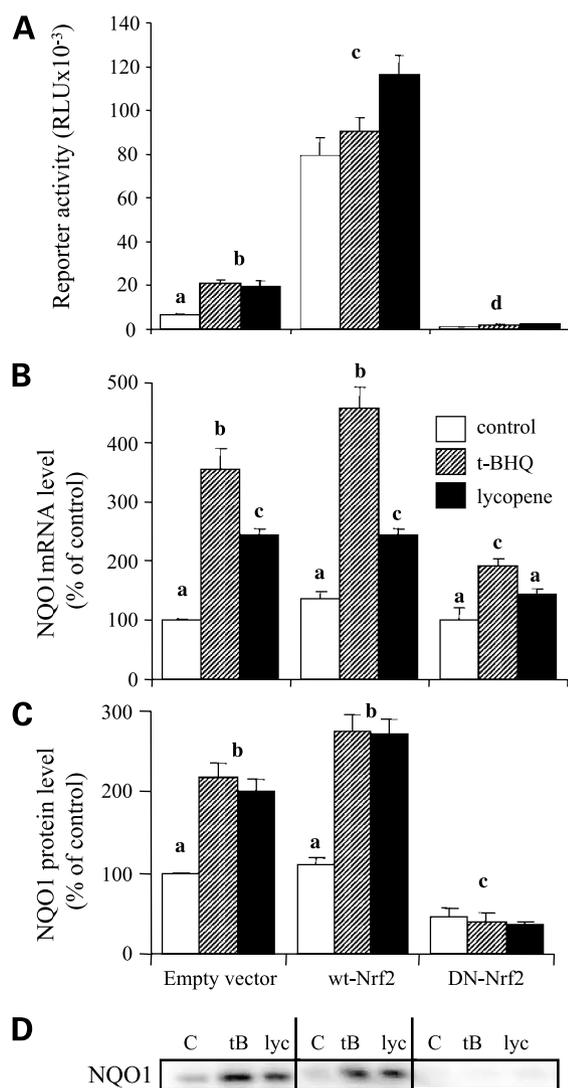
Figure 3. Effects of a dominant-negative Nrf2 on ARE transactivation (**A**) and the induction of NQO1 mRNA (**B**) and protein (**C**) by lycopene. **A,** MCF-7 cells were transiently cotransfected with NQO1hARE-*tk-luc* reporter plasmid and either a dominant-negative Nrf2 (*DN-Nrf2*), wild-type Nrf2 (*wt-Nrf2*) or the appropriate empty vector. The effect of tBHQ and lycopene on the reporter gene activity was analyzed as described in Fig. 1. The levels of mRNA (**B**) and protein (**C**) were measured as described in Fig. 2, in a mixture of HepG2 cells stably transfected with either the DN-Nrf2, wt-Nrf2, or the appropriate empty vector. Results are expressed as a percentage of mRNA or protein level in control (tetrahydrofuran) cells that were transfected with the empty vector. Mean (*columns*) ± SE (*bars*) of two to three independent experiments. Differences among the multiple groups were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Columns not sharing a common letter are statistically significantly different at $P < 0.05$.

found hydrophilic oxidized derivatives of this carotenoid from the parent molecule. Indeed, this ethanolic extract transactivated the ARE reporter genes, in a dose-dependent manner, at a potency which was equivalent to that of the original lycopene preparation (Fig. 4A). The same extract also induced the expression of NQO1 protein in HepG2 cells (Fig. 4B).

We examined whether the dominant-negative Nrf2 reduces the ability of the lycopene ethanolic extract to stimulate ARE transcriptional activity and to increase the protein levels of phase II enzymes. Indeed the transactivation of ARE and the increase in NQO1 protein level was totally abolished in cells transfected with the dominant-negative Nrf2 (results not shown).

Nrf2 Nuclear Translocation and Colocalization with PML Protein

Studies using immunostaining and other methods have shown that activation of transcription through ARE by various agents is preceded by nuclear translocation of the transcription factor Nrf2 (29). Thus, we investigated by immunostaining whether carotenoid treatment results in translocation of Nrf2 from the cytosol to the nucleus. HepG2 cells were treated with tBHQ (50 µmol/L) or various carotenoids (3–10 µmol/L) for 5 hours. Nrf2 localization was visualized using an anti-Nrf2 antibody and Alexa Fluor 488-conjugated secondary antibody (Fig. 5A, *left*). 4′,6-Diamidino-2-phenylindole staining of the same cells identified the nucleus localization (Fig. 5A, *right*). Nrf2 was found to be predominant in the cytoplasm of nontreated (control) cells (Fig. 5A, *a*), but after treatment with tBHQ (Fig. 5A, *b*) or lycopene (Fig. 5A, *c*) was predominant in the nucleus. Other carotenoids also caused Nrf2 translocation but with a lower potency (Fig. 5A, *d–f*).

It has previously been shown that several transcription factors, such as p53 and various steroid receptors, colocalize with the PML protein in PML nuclear bodies (36), thereby modulating their transcriptional activity (37). Therefore, we used double immunolabeling to study the colocalization of PML and Nrf2 proteins as a function of
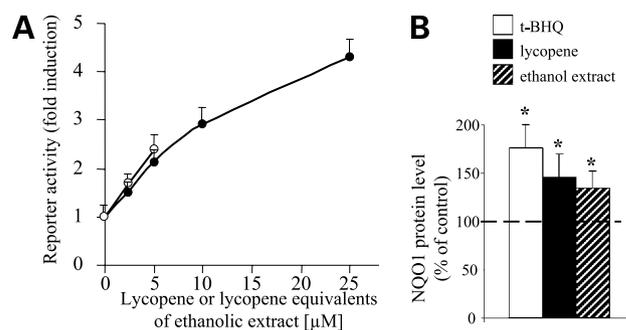

Figure 4. Effects of ethanolic extract of lycopene on ARE transactivation (**A**) and the levels of NQO1 protein (**B**). HepG2 cells were treated with ethanolic extract of lycopene prepared as described under Materials and Methods. **A,** the effect of lycopene or its ethanolic extract on the reporter gene activity was analyzed as described in Fig. 1. The total concentration of the compounds in the ethanolic extract is presented as the equivalent of the starting amount of lycopene. **B,** the effect of 30 µmol/L tBHQ, 5 µmol/L lycopene, or an equivalent concentration of an ethanolic extract of lycopene on the level of NQO1 protein was measured as described in Fig. 2. Mean (*columns*) ± SE (*bars*) SE of two to three independent experiments. *, values that are significantly different ($P < 0.05$) from the control (*dashed line*).
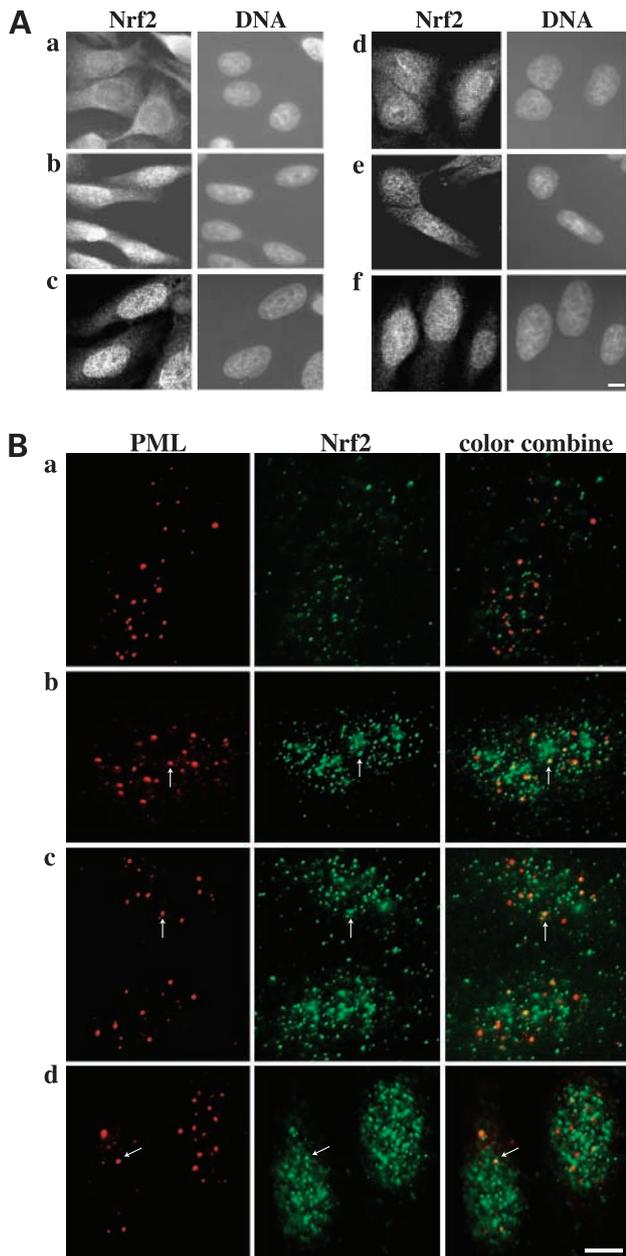
Figure 5. Effects of carotenoids on Nrf2 translocation to the nucleus (**A**) and localization to PML nuclear bodies (**B**). **A,** HepG$_2$ cells were exposed to tetrahydrofuran (*a*), 50 μmol/L tBHQ (*b*), lycopene (*c*), astaxanthin (*d*), phytoene (*e*), and β-carotene (*f*). Carotenoid concentrations are indicated in Fig. 1. After 5 h, cells were fixed and processed for immunostaining as described under Materials and Methods. Nrf2 protein (*left*) was visualized by 488 Alexa Fluor-conjugated secondary antibodies, whereas DNA (*right*) was visualized by 4′,6-diamidino-2-phenylindole staining. Representative fields are shown. *Bar,* 10 μm. **B,** HepG$_2$ cells were exposed to ethanol (*a*), 50 μmol/L tBHQ (*b*), lycopene (*c*), or β-carotene (*d*) and processed for double Nrf2 and PML immunostaining. Nrf2 localization (*middle*) was visualized as in **A**, using the *green channel* of the fluorescence microscope, whereas PML localization (*left*) was visualized by 568 Alexa Fluor-conjugated secondary antibodies, using the *red channel* of the fluorescence microscope. *Right,* color-combined images of Nrf2 and PML protein staining in which Nrf2 is represented by *green* and PML by *red* colors. In the combined images, *orange* and *yellow* colors represent areas in which Nrf2 and PML proteins are colocalized (*white arrows*). *Bar,* 10 μm.

stimulation with tBHQ, lycopene, and β-carotene (Fig. 5B). In agreement with previous reports (36), we found that the PML protein is organized in distinct areas which most likely represent the PML nuclear bodies (Fig. 5B, *left*). To visualize PML and Nrf2 protein colocalization, we used color-combined images in which the Nrf2 protein is represented by *green* (Fig. 5B, *middle*), PML protein is represented by *red* (Fig. 5B, *left*), and areas of colocalization are visualized by *orange* and *yellow* (Fig. 5B, *right*). We found that 4- to 6-hours' treatment with tBHQ, lycopene, and β-carotene significantly increased the number of PML bodies in which the Nrf2 protein is present (Fig. 5B, *right, arrows*). Whereas in control cells, the number of PML bodies containing Nrf2 protein was negligible, zero to one per cell (Fig. 5B, *a*), after stimulation their number increased to three to eight per cell (Fig. 5B, *b–d*). The percentage of Nrf2-containing PML nuclear bodies increased from 0% to 5% in control cells and 30% to 70% in tBHQ or carotenoid-treated cells. These data clearly show that tBHQ, lycopene, and β-carotene not only translocate Nrf2 protein to the nucleus (Fig. 5A), but also cause its localization to the PML nuclear bodies (Fig. 5B). However, it is not clear whether the localization in the nuclear bodies has a role in regulating Nrf2 activity.

Effect of Carotenoids on Intracellular Levels of GSH and Reactive Oxygen Species

Because carotenoid treatment resulted in the induction of GCS (Fig. 2), the rate-limiting enzyme in glutathione synthesis (25), using 5,5-dithiobis-(2-nitrobenzoic acid) colorimetric assay, we examined whether GSH levels would also increase under these conditions. *N*-acetylcysteine, a nonspecific thiol antioxidant, and buthionine sulfoximine, a specific GCS inhibitor, were used as positive and negative controls, respectively (Fig. 6A). We found that tBHQ, lycopene, and β-carotene elevated GSH level in HepG$_2$ cells (Fig. 6A), whereas phytoene and astaxanthin had no effect. The increase in GSH level by tBHQ and lycopene correlates well with their effect on the induction of GCS shown in Fig. 2.

Carotenoids are well known for their antioxidant activity. To examine whether the potency of these compounds in activating ARE correlates with their antioxidant activity, we measured their effects on intracellular reactive oxygen species (ROS) levels under the same experimental conditions which were used for analysis of ARE activation. ROS level was monitored using dichlorofluorescein fluorescence (38). We found that treatment of HepG$_2$ and MCF-7 cells with tBHQ, lycopene, phytoene, or astaxanthin lowered intracellular ROS levels to a similar extent (Fig. 6B), which did not correlate with the abilities of these compounds to activate ARE. Thus, tBHQ and lycopene, which were much more active than the other agents in activating ARE (see Fig. 1), decreased intracellular ROS levels in HepG$_2$ cells with nearly the same potency as phytoene or astaxanthin (Fig. 6B). On the other hand, although β-carotene did not reduce intracellular ROS levels, its potency to transactivate the ARE reporter gene

was similar to that of phytoene, phytofluene, and astaxanthin. These data indicate that the ability of carotenoids to transactivate ARE is not solely related to their antioxidant activity.

Discussion

The main finding of the present study is that lycopene, and to a lesser extent other carotenoids, induce phase II enzymes through activation of the ARE transcription system, which may explain some of the cancer-preventing activity of carotenoids. We found that lycopene transactivated transcription from the promoters of the two phase II enzymes, NQO1 and GCS. These findings correlate with increased expression of the two enzymes and the nuclear translocation of the Nrf2 transcription factor. The induction of these phase II enzymes by tBHQ and lycopene, was totally abolished in the presence of dominant-negative Nrf2 lacking the transactivation domain. For the present, there is no clear explanation why lycopene was more potent than all the other tested carotenoids in activating ARE. Although lycopene consistently activated all the measured parameters, the effects of the other carotenoids were not consistent. For example, β-carotene induced NQO1 expression and Nrf2 nuclear translocation but showed only a marginal effect on ARE transactivation and intracellular GSH levels. Differences in carotenoid uptake across the cell membrane probably cannot account for the differences in carotenoid activity because absorption of β-carotene was by far better than that of lycopene in a similar *in vitro* system using the intestinal cancer cell line, Caco-2 (39). ARE activation was not related directly to the antioxidant function of the carotenoids. This conclusion is based on the findings that most of the tested carotenoids similarly lowered intracellular ROS levels but varied significantly in their potency to activate ARE.

Interestingly, part of the translocated Nrf2, colocalized with the PML protein in the PML nuclear bodies. To date, more than 40 different cellular proteins have been found to be associated with PML nuclear bodies (36). Although the molecular function of PML nuclear bodies is currently not clear, there is accumulating evidence that they are regulatory domains involved in various biological processes (36), including cell growth, apoptosis, cellular senescence, DNA repair, antiviral response, protein degradation, and more relevant to the current study, transcriptional regulation (40). Thus, our finding that lycopene, β-carotene, and tBHQ cause the localization of Nrf2 in PML nuclear bodies may point to the role of these nuclear bodies in regulating phase II enzyme expression by Nrf2. More studies are needed in order to understand the relevance of lycopene-induced localization of Nrf2 in PML bodies and the role of a higher-order architecture of the cell nucleus in cancer prevention by carotenoids and other dietary compounds.

Our data allow adding carotenoids to a wide range of diet-derived compounds, such as curcuminoids, coumarins, diterpenes, dithiolethiones, isothiocyanates, organo-

sulfides, and polyphenols (19, 41), all of which activate ARE. One of the active chemical groups common to many of these compounds is the electrophilic α,β-unsaturated carbonyl (21). It is clear that such a chemical group is not part of the intact carotenoid molecule, but rather may exist in cleavage and oxidation derivatives of carotenoids. Indeed, we found that the ethanolic extract of lycopene containing unidentified hydrophilic derivatives of the carotenoid activates ARE with a similar potency to lycopene. The question of whether intact lycopene is devoid of such activity remains open, as the half-life of lycopene in the culture medium is < 1 day (8, 42), and thus its active oxidation products may also be formed during the experiment. This degradation may be prevented, e.g., by coincubation with another antioxidant. However, the addition of a protective antioxidant or another degradation
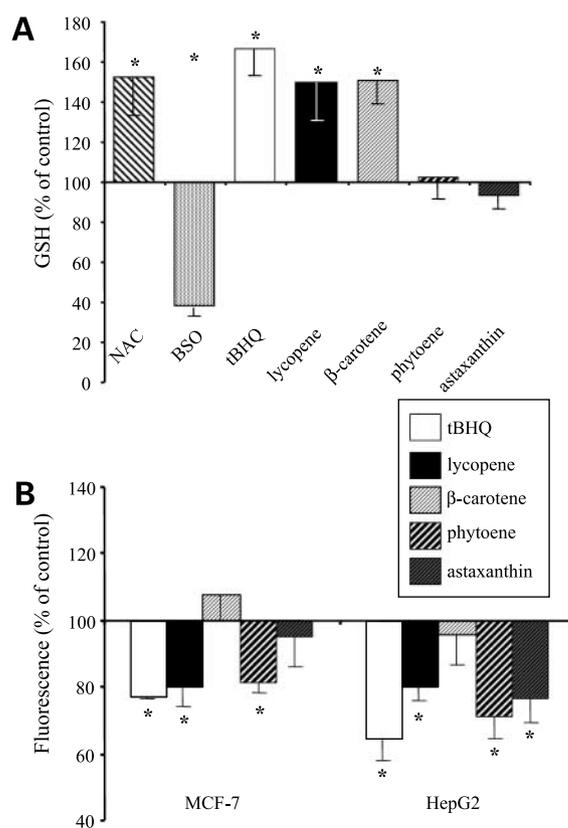

Figure 6. Effects of carotenoids on intracellular levels of GSH and ROS. HepG$_2$ cells (**A** and **B**) and MCF-7 cells (**B**) were treated for 24 h with carotenoids as described in Fig. 2. **A,** intracellular levels of GSH were measured by a colorimetric assay using 5,5-dithiobis-(2-nitrobenzoic acid) as described under Materials and Methods. *N*-acetylcysteine (10 mmol) and buthionine sulfoximine (100 μmol/L), were used as positive and negative control, respectively. **B,** levels of intracellular ROS were measured using DCF fluorescence as described under Materials and Methods. The fluorescence data were normalized to protein content in the samples. Results are expressed as a percentage of GSH level or fluorescence in control (tetrahydrofuran) cells; mean (*columns*) ± SE (*bars*) six to seven independent experiments, each done in duplicate (**A**) or four to ten replicates (**B**). *, values that are significantly different (*P* < 0.05) from the control (100%).

blocker *per se* would affect cellular functions, e.g., by changing the redox status of the cell which may add to the carotenoid effect. A putative lycopene derivative, *acyclo-retinoic acid*, was previously reported by us (43) and others (44, 45) to activate transcription by the retinoic acid receptor. Recently, another oxidized derivative of lycopene which activates gap junctional communication was identified as a dialdehyde derivative of the carotenoid, having α, β-unsaturated carbonyl groups (46). Furthermore, lycopene derivatives having α,β-unsaturated carbonyl groups or other electrophilic groups were formed after spontaneous and induced chemical oxidation of the carotenoid (47–51). We are currently attempting to characterize derivative(s) of lycopene and other carotenoids which activate ARE. To assess whether such electrophilic derivatives are physiologically relevant, it is important to know if they can be formed in the human body and which metabolic pathways lead to their formation. One study in this direction, using the postmitochondrial fraction of rat intestinal mucosa, showed the formation of lycopene metabolites in a biological system (52).

A link between the induction of phase II enzymes and the cancer-preventive effects of various phytonutrients including carotenoids, such as β-carotene, was suggested in 1981 by Peto et al. (53), however, since then *in vivo* studies with carotenoids have produced ambivalent results. For example, Gradelet et al. (23) have shown that canthaxanthin and astaxanthin, but not lutein and lycopene, are capable of inducing *p*-nitrophenol-UDP-glucuronosyl transferase and NQO1 in rat liver. On the other hand, in a similar study in rat liver, Breinholt et al. (24) showed that lycopene induced the expression of several phase II enzymes, including NQO1. Bhuvaneswari et al. (54) showed an association between the reduction in the incidence of 7,12-dimethyl-benz(*a*)anthracene-induced hamster buccal pouch tumors by lycopene and a concomitant increase in the level of GSH, the phase II enzyme glutathione *S*-transferase and enzymes of the glutathione redox cycle. Based on these results, the authors suggested that the lycopene-induced increase in the levels of GSH and the phase II enzyme glutathione *S*-transferase in the buccal pouch mucosa can inactivate carcinogens by forming conjugates that are less toxic and readily excreted from the body (54). In the present study, we also show that lycopene increases GSH levels in cancer cells and provide the likely mechanism for this effect based on the carotenoid-induced nuclear translocation of Nrf2, activation of ARE in the promoter of GCS and the induction of the enzyme protein, which would ultimately lead to the elevated *de novo* synthesis of glutathione. Bertram et al. (55) also reported that lycopene and other carotenoids are able to inhibit the early stages of the cancer process. These authors showed that carotenoids inhibited the production of transformed foci, an established marker for malignant transformation, when given to methylcholanthrene-treated 10T1/2 cells.

In conclusion, we report here that the induction of phase II enzymes, such as NQO1 and GCS, in mammary and liver cancer cells by lycopene and other carotenoids, is regulated by the ARE transcription system. Collectively, the data presented in this paper are consistent with the specific requirement of intact Nrf2 transcription factor in the activation of cancer-preventive genes by carotenoids. Because lycopene and other carotenoids have been shown to reduce risk for several malignancies in humans, the activation of ARE provides a molecular mechanism for the role of a carotenoid-rich diet in cancer prevention.

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