Pivotal Role of Electrophilicity in Glutathione *S*-Transferase Induction by *tert*-Butylhydroquinone†

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Received January 6, 2003; Revised Manuscript Received February 14, 2003

ABSTRACT: Although the induction of glutathione *S*-transferase (GST) activity by *tert*-butylhydroquinone (tBHQ) has been well-documented in several cell culture systems and rodent experiments, the exact mechanism responsible for its inducibility is still not thoroughly understood. To more precisely define the molecular mechanism of GST induction by tBHQ, we examined the one-electron oxidation and glutathione (GSH) reaction potentials of tBHQ as compared to its analogue, 2,5-di-*tert*-butylhydroquinone (DtBHQ). tBHQ and DtBHQ showed similar one-electron oxidation potentials, including free radical quenching (antioxidant), oxidative conversion of both compounds to a benzoquinone form, and Cu^{2+}-dependent superoxide generation. On the other hand, the reduced GSH level was observed by the addition of tBHQ, but not DtBHQ, suggesting that tBHQ acts as an electrophile while DtBHQ does not. The data were consistent with the observation that tBHQ more potently induced the *GSTP1* gene expression in RL34 cells than DtBHQ did. Moreover, we indeed detected the GSH-tBHQ conjugates in the cells exposed to tBHQ using an electrochemical detector−high-performance liquid chromatography technique. Thus, we conclude that an electrophilic quinone oxidation product that reacts with intracellular nucleophiles including protein thiol or GSH plays a major role in the *GSTP1* gene expression.

Phase II enzymes such as NAD(P)H:(quinone-acceptor) oxidoreductase (NQO[1]) and glutathione *S*-transferase (GST) play a role in the cellular detoxification of genotoxic and carcinogenic chemicals. The GSTs are a family of enzymes that catalyze the nucleophilic addition of the thiol group of reduced glutathione (GSH) to a variety of electrophiles (for review, see ref *1*). It is generally accepted that the GSTs are encoded by at least five different gene families. Four of the gene families (Class alpha, mu, pi, and theta) encode the cytosolic GSTs, whereas the fifth encodes a microsomal form of the enzyme. Recently, two transgenic rodent studies clearly demonstrated that the Class pi GST (*GSTP1*) can profoundly alter the susceptibility to chemical carcinogenesis in mouse skin (*2*) and rat liver (*3*). The Class pi rat and human GST isozymes have been shown to be highly efficient in the GSH conjugation of carcinogenic benz[*a*]pyrene derivatives (*4*, *5*), widespread environmental pollutants in cigarette smoke, and automobile exhaust. In addition, GSTP1-1 is more effective in the detoxification of electrophilic α,*β*-unsaturated carbonyl compounds produced by radical reactions, lipid peroxidation, ionizing radiation, and the metabolism of drugs than other GSTs (*6*). Thus, the induction of GSTP1-1 is regarded as one of the important determinants in the cancer chemoprotection potential of food stuffs, phytochemicals, and synthetic chemicals.

An antioxidant/electrophile response element (ARE/EpRE; consensus sequence TGACNNNGC) or the related element, regulating both its basal and inducible expressions, was mostly found in the 5′-flanking region of the genes of phase II enzymes and may be recognized by a similar series of transcriptional factors (*7*). Recently, we developed the cultured hepatocyte cell line RL34 and determined the GST induction potencies of edible plants, structures, and their molecular mechanism of several series of compounds (*8*−*12*). Using this cell line, we demonstrated that for the first time that *GSTP1* enhancer I (GPEI; ARE/EpRE of GSTP1), containing a palindromic dyad of the 12-*O*-tetradecanoylphorbol-13-acetate responsible element (TRE)-like sequence (*13*), is an essential cis-element required for the activation of the GSTP1 gene through the redox alteration by electrophiles such as benzyl isothiocyanate or diethyl maleate (DEM) (*9*).

† This study was supported by grants-in-aid for Encouragement of Young Scientists (B) (No. 13760102) and in part by Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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[1] Abbreviations: NQO, NAD(P)H:(quinone-acceptor) oxidoreductase; GST, glutathione *S*-transferase; GSH, glutathione; ARE/EpRE, antioxidant/electrophile response element; GPEI, *GSTP1* enhancer I; TRE, 12-*O*-tetradecanoylphorbol-13-acetate responsible element; DEM, diethyl maleate; tBHQ, *tert*-butylhydroquinone; ROS, reactive oxygen species; BQ, 1,4-benzoquinone; BHA, 3-*tert*-butyl-hydroxyanisole; DtBHQ, 2,5-di-*tert*-butylhydroquinone; H_2DCF-DA, 2′,7′-dichlorofluorescin diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DCF, dichlorofluorescein; NMR, nuclear magnetic resonance; SOD, superoxide dismutase; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; CAT, chloramphenicol acetyltransferase; ERK, extracellular signal-regulated protein kinase; MKK3, mitogen actived protein kinase kinase 3; Nrf2, NF-E2-related factor 2.

10.1021/bi0340090 CCC: $25.00
Published on Web 03/22/2003

The findings of chemical agents having diverse structures indicated that various chemicals may produce a common signal transduction responsible for AP-1 or its related factor-(s). It has been observed that most chemical inducing agents of the phase II enzymes have the potential to induce oxidative stress and depletion of the reduced GSH (*9*, *14*).

Phenolic compounds, termed phenolic antioxidants because of their chain-breaking reaction during autoxidation of lipids, are utilized for food preservation and the suppression of lipid peroxidation in biological materials. Previous studies, based on the assumption that these antioxidative properties are important for the inducible effect on the AP-1-mediated gene expression including phase II enzymes, have concluded that the AP-1 binding site is an antioxidant response element. Concerning the molecular basis of cancer protection by *tert*-butylhydroquinone (tBHQ), Talalay and his colleagues also concluded in their studies of the induction of phase II enzymes that oxidative lability was essential for inducer activity since catechol (1,2-diphenols) and hydroquinone (1,4-diphenols) derivatives undergo facile oxidation to quinones, whereas 1,3-diphenols, inactive for phase II induction, cannot participate in such an oxidation (*15*). Although these experiments did not establish whether the oxidation products or oxidation processes (potentially involving radical scavenging reaction, multiple one- and two-electron oxide reduction, and redox-dependent reactive oxygen species (ROS) generation) were an inductive signal, electrophilic quinone oxidation products were presumed to be the ultimate inducers since electrophiles including Michael reaction acceptors (e.g., olefins conjugated to electron-withdrawing groups) and isothiocyanates potentially induce the phase II enzyme expression (*16*). Conversely, Pinkus et al. demonstrated that the autoxidation of tBHQ to the semiquinone radical or 1,4-benzoquinone (BQ) and the generation of hydroxyl radical were detected using the electron spin resonance spectroscopy technique (*17*). They also showed that the induction of an endogenous GST alpha class gene (*rGSTA1*) in hepatoma cells by tBHQ was inhibited by antioxidants *N*-acetylcysteine, GSH, and exogenous catalase. It was thus expected that the intermediate formation of H_2O_2 during the metabolism of tBHQ may be a critical step for the phase II enzyme induction. However, the dose-dependency of the hydroxyl radical generation was poorly correlated with the GST gene expression; as the concentration of tBHQ increased, a lower amount of hydroxyl radical was generated (*17*). More recently, Lee et al. pointed out that the ROS-generating property can be ruled out in the mechanism for the ARE-dependent gene expression by tBHQ (*18*). Anyway, phenolic antioxidants express antagonistic signals, oxidative stress, and antioxidative reactions in the cells.

tBHQ is a major metabolite in vivo in dogs, rats, and man of 3-*tert*-butyl-hydroxyanisole (BHA) (*19*−*21*), a synthetic phenolic antioxidant frequently used as a food additive. BHA protects animals against various carcinogens, presumably through the induction of many phase II detoxifying enzymes as well as the inhibition of cytochrome P450 monooxygenase. On the other hand, mounting evidence has indicated that BHA can be a carcinogen or tumor promoter in some tissue in animals. Although opposing biological effects of BHA on carcinogenesis have been well-documented, the precise mechanisms of these effects remain obscure. tBHQ has also been reported to be a double-edged sword in cancer control, possibly through phase II enzyme induction including GST activity and through the generation of ROS by cytochrome P450/P450 oxidoreductase- or transition metal-mediated redox cycling, respectively. Thus, the metabolic formation of tBHQ is thought to, at least in part, contribute to the modifying effects of BHA on carcinogenesis.

In the present study, we further investigated the role of the redox cycling reaction or electrophilic property of tBHQ in the induction of GST. GST activity, intracellular oxidative stress, and GSH consumption were monitored in intact RL34 cells or in the cell-free system applied to tBHQ and 2,5-di-*tert*-butylhydroquinone (DtBHQ), derived from one of the concomitants of commercial BHA, 2,5-di-*tert*-butylhydroxyanisole (*22*), with similar potentials to construct redox cycling and different capacities to react with nucleophiles such as GSH. We demonstrated for the first time that tBHQ is a potential inducer of pi class GSTP1-1 isozyme, an important molecule that protects against carcinogens as described above and that GPEI is the responsible element in the *GSTP1* induction by tBHQ. We report here that DtBHQ, hardly reacting with GSH probably because of steric hindrance of the bulky *tert*-butyl moieties, has much less ability for GST induction. Thus, we conclude that the reaction with an intracellular nucleophile including protein thiol or GSH is virtually responsible for the induction of GSTP1-1 and/or other phase II enzymes by tBHQ.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan. Anti-rat GSTP1-1 antisera was obtained from Biotrin International, Dublin, Ireland. Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin was purchased from Dako, Glostrup, Denmark. 2′,7′-Dichlorofluorescin diacetate (H_2DCF-DA) was obtained from Molecular Probes, Inc., Leiden, The Netherlands. The protein concentration was measured using the BCA protein assay reagent from Pierce. Authentic tBHQ-GSH conjugates were synthesized as previously reported (*23*).

Cell Cultures. RL34 cells was obtained from the Health Science Research Resources Bank, Osaka, Japan (*24*). The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (0.3 mg/mL), pyruvic acid (0.11 mg/mL), and 0.37% $NaHCO_3$ at 37 °C in a atmosphere of 95% air and 5% CO_2. Cells post-confluency were exposed to the test compounds in a medium containing 5% fetal bovine serum.

Radical Scavenging Activity Assay. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was evaluated as previously reported (*25*). A test compound mixed with a 100 mM Tris-HCl buffer (pH 7.4, 1 mL) was added to 0.5 mM DPPH in ethanol (1 mL), and the mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The DPPH radical scavenging activity is expressed as the ratio of the relative decrease in the absorbance of the test sample mixture at 517 nm to that of the 1 mM Trolox solution: DPPH radical scavenging activity (%) = {(vehicle) − (test compound)}/{(vehicle) − (Trolox)} × 100.

Intracellular Peroxide Determination. Intracellular peroxides were detected by H_2DCF-DA as an intracellular

fluorescence probe (*9*, *26*). Briefly, the cells under confluency were preincubated with tBHQ and DtBHQ (50 μM) for 1 h. After stimulation by DEM (0.5 mM) for 15 min and washing by PBS, the cells were treated with H_2DCF-DA (50 μM) for 30 min at 37 °C. A flowcytometer (CytoACE 150, JASCO, Tokyo, Japan) was used to detect dichlorofluorescein (DCF) formed by the reaction of H_2DCF with intracellular oxidative products. Experiments were repeated four times with similar results. The data are expressed as one representative histogram.

GSH Assay. Measurement of the GSH level was performed spectrophotometrically using commercial kit GSH-400 (BIOXYTECH) (*27*). GSH (0.1 mM) was incubated with tBHQ or DtBHQ (0.1 mM) in 100 mM sodium phosphate buffer, pH 7.4, at 25 °C, gently in the dark. Fifty microliters of 12 mM chromogenic reagent in 0.2 M HCl was added to the reaction mixture (300 μL) at the end of incubation periods and mixed thoroughly. After 50 μL of 7.5 M NaOH was added and mixed, the mixture was incubated at 25 °C for 10 min, and then absorbance was determined spectrophotometrically at 400 nm.

NMR Experiment. Nuclear magnetic resonance (NMR) was performed using a Bruker ARX-400 spectrometer with tetramethylsilane as the internal standard.

Measurement of O_2^- Generation. The quantity of O_2^- generated by the reaction of tBHQ or DtBHQ with Cu^{2+} was determined by cytochrome *c* reduction as previously reported (*28*). The reaction mixture, containing 50 μM ferricytochrome *c*, 20 μM tBHQ or DtBHQ, 20 μM Cu^{2+}, and 5 μM DTPA in 1.2 mL of 100 mM sodium phosphate buffer (pH 7.4) with or without superoxide dismutase (SOD, 150 units/mL), was incubated at 37 °C. We recorded the absorption at 550 nm spectrophotometrically.

HPLC-ECD Analysis. A HPLC system equipped with a PU-980 HPLC pump, 807-IT Integrator, and both UV-970 UV and 840-EC electrochemical detectors was used. Elution profiles were monitored at 210 nm on the UV detector and at 0.5 V of the applied oxidation potential on the electrochemical detector. A reverse-phase column (NOMURA CHEMICAL, Aichi, Japan; Develosil ODS-HG-5; 25 × 0.8 cm) was used throughout this study. The tBHQ-GSH conjugates formed intracellularly were measured by this system. Confluent monolayer cells were exposed to 10 μM tBHQ, and after the various incubation periods, cell monolayers were washed twice PBS (pH 7.0) and extracted with the 5% trichloroacetic acid solution containing 5 mM EDTA followed by centrifugation (10 000*g*, 20 min). The column was equilibrated, and the cell extracts were eluted with 55% MeOH/water containing 0.1% formic acid at a flow rate of 0.8 mL/min. The identification of the conjugates was made on the basis of the retention time of authentic samples as well as on the coelution test performed by adding standard conjugates to cell samples.

Enzyme Assay. GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) as substrates according to the method of Habig and Jakoby (*29*).

Western Blot Analysis. For GST, the tBHQ derivatives treated and untreated cells were rinsed twice with PBS (pH 7.0) and lysed by incubation at 37 °C for 10 min with a solution containing 0.8% digitonin and 2 mM EDTA (pH 7.8). Each whole cell lysate was then treated with Laemmli sample buffer for 3 min at 100 °C (*30*). The samples (20 μg) were run on a 12.5% SDS−PAGE slab gel. One gel was used for staining with Coomassie brilliant blue, and the other was transblotted on a nitrocellulose membrane with a semi-dry blotting cell (Trans-Blot SD, Bio-Rad) incubated with Block Ace (40 mg/mL) for blocking, washed, and treated with the antibody.

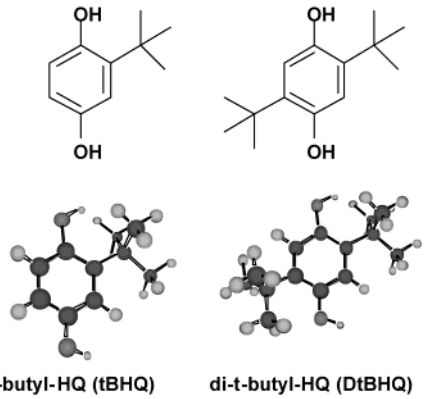


FIGURE 1: Chemical and stereoscopic structures of tBHQ and DtBHQ.

Plasmid Construction. Chloramphenicol acetyltransferase (CAT) fusion plasmids were constructed by the method of Sakai et al. (*13*) and were a kind gift from Prof. M. Imagawa of Nagoya City University. A 3.0 kb fragment between −2.9 kb and +59 bp of the gene for GSTP1-1 (*31*, *32*) was inserted into the *Hin*dIII site of pSV0CAT (*33*) and designated ECAT. A series of 5′ deletion mutants were constructed from the ECAT using appropriate restriction enzymes (*13*).

DNA Transfection for Analysis of CAT Activity. RL34 cells were transfected with 5 μg of plasmid construct by a calcium phosphate coprecipitation procedure described by Chen and Okayama (*34*). The test compound was added to the culture medium 48 h after transfection. Cell lysates were obtained after freeze−thawing three times in 0.25 M Tris-HCl (pH 7.4) and used for the CAT assay. For the CAT assay (*35*), the extracts were heated at 65 °C for 10 min, and the precipitates were removed by centrifugation at 15 000*g* for 10 min at 4 °C. The reaction mixtures (final volume 125 μL) containing the cell extract, 210 mM Tris-HCl (pH 7.8), 11 kbp of 1-deoxy[^{14}C]chloramphenicol, and 80 nmol of acetyl-CoA were incubated at 37 °C for 90 min. Reactions were terminated by the addition of 1 mL of ethyl acetate. The product (3-acetyl-1-deoxychloramphenicol) and the unreacted substrate were extracted with ethyl acetate. After ethyl acetate was evaporated, the residue was disolved in 20 μL of ethyl acetate and chromatographed on a thin-layer plate with chloroform/methanol (95:5, v/v). The radioactivity of the product and substrate was analyzed using the Fuji BAS 2000 system (Fuji Photo, Tokyo).

RESULTS

Redox Cycling Potentials of tBHQ and DtBHQ. Quinones shuffle electrons enzymatically or nonenzymatically among their reduced form (hydroquinone), oxidized form (BQ), and/or their semiquinone radicals to construct redox cycles. To investigate the redox process, we first examined the free radical scavenging activity of tBHQ and DtBHQ because the scavenging of a free radical is completed by the one-electron oxidation of the parent compound. As shown in Figure 2A, the dose-dependent effect to scavenge a well-

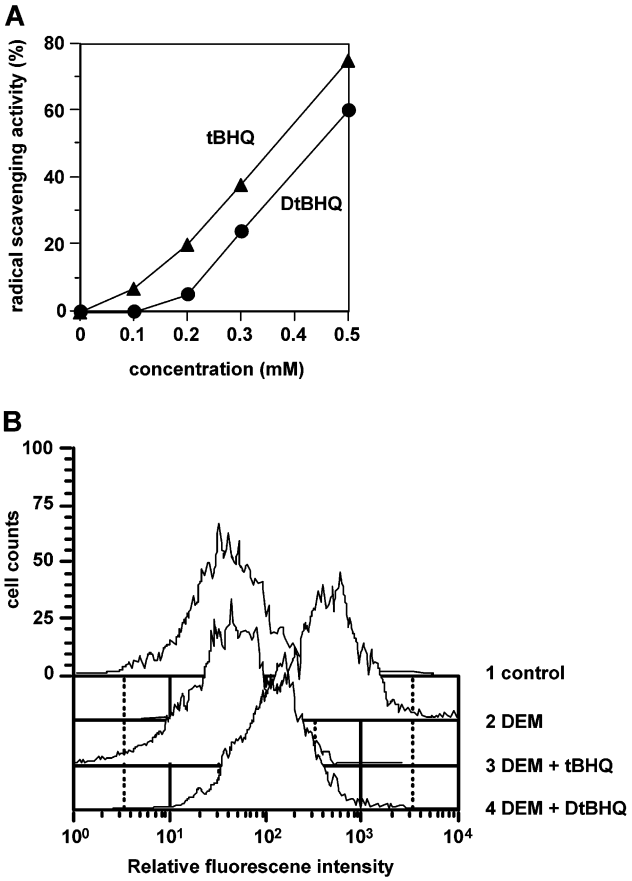


FIGURE 2: tBHQ and DtBHQ act as antioxidants in the cell-free and cultured cell systems. (A) Free radical scavenging activity against DPPH radical of tBHQ (closed triangle) and DtBHQ (closed circle). A test compound mixed with a 100 mM Tris-HCl buffer (pH 7.4, 1 mL) was added to 0.5 mM DPPH in ethanol (1 mL), and the mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The DPPH radical scavenging activity is evaluated by the absorbance at 517 nm. (B) Inhibitory activity of tBHQ and DtBHQ against DEM-induced intracellular ROS generation in RL34 cells. The cells were pretreated with tBHQ and DtBHQ (50 μM) for 1 h and then stimulated with 0.5 mM DEM for 30 min. The cells were incubated with H_2DCF-DA (50 μM) for 30 min to detect intracellular peroxide formation. The DCF fluorescence of more than 10 000 cells was monitored on a flowcytometer.

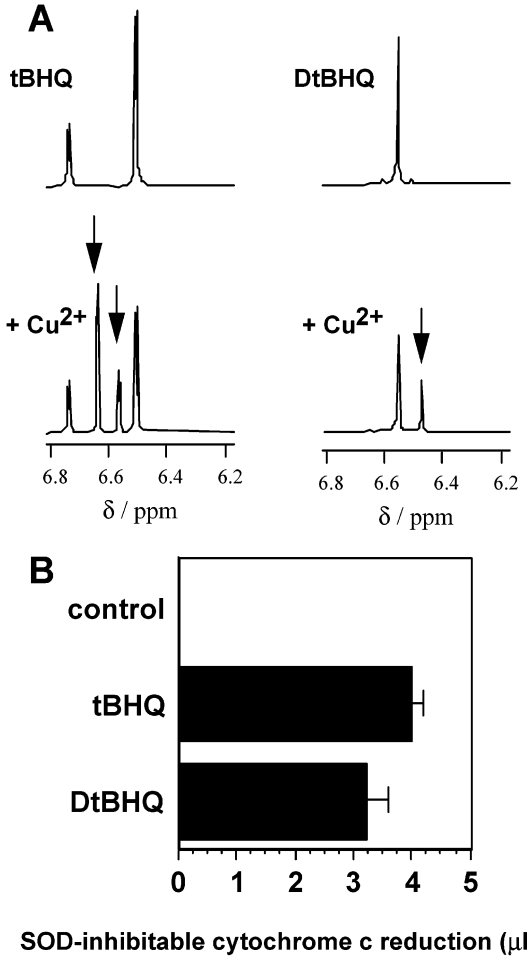


FIGURE 3: Redox reactions of tBHQ and DtBHQ. (A) ^{1}H NMR spectra of tBHQ (left) or DtBHQ (right) treated with or without Cu^{2+}. An aqueous solution (0.75 mL) containing 27 mM DtBHQ (upper) or DtBHQ, 27 mM $CuCl_2$, and 27 mM bathocuproine disulfonic acid (lower) was shaken vigorously for 1 min. The organic compounds were extracted in chloroform-*d* to measure the spectrum. The arrow points to a signal assigned for oxidized DtBHQ (2,5-di-*tert*-butylbenzoquinone). (B) Cytochrome *c* reduction by tBHQ and DtBHQ with Cu^{2+}. Reactions were performed with 10 μM tBHQ or DtBHQ plus 10 μM $CuCl_2$ and 50 μM cytochrome *c* with or without 150 units/mL SOD in 1.2 mL of 100 mM phosphate buffer (pH 7.4) with DTPA. The amount of O_2^- genaration was estimated by subtracting the amount of reduced cytochrome *c* with SOD from that without SOD.

known free radical, DPPH, was observed when the DPPH radical and DtBHQ were mixed in a solution. This activity was relatively less than that of tBHQ but significant. Thus, it appears that DtBHQ as well as tBHQ can be oxidatively converted to the corresponding semiquinone radical (one-electron oxidation product) or BQ (fully oxidized by two electrons).

Next, to determine whether tBHQ and DtBHQ can act as an antioxidant within cells, we investigated the inhibitory effect during the short exposure of electrophile-induced intracellular ROS generation using a flowcytometry technique (*36*). As shown in Figure 2B, the cells treated for 30 min only with the strong thiol blocker DEM exhibited a significant accumulation of DCF because of the DEM-induced ROS production. Pretreatment of tBHQ significantly blocked the DEM-induced enhancement of the intracellular ROS accumulation. DtBHQ also reduced the intracellular ROS production. Treatment only with tBHQ or DtBHQ did not affect the basal level of accumulated DCF (data not shown). The intracellular antioxidant activity of DtBHQ is weaker than that of tBHQ as expected by the result in the DPPH assay. These results suggested that tBHQ and DtBHQ could be oxidized to the corresponding semiquinone radical or benzoquinone in biological systems. Because the exact mechanism responsible for their antioxidant activity against intracellular ROS accumulation is still not thoroughly understood, further studies of ROS molecule or free radical species contributed to DEM-induced oxidative stress are required.

To gain further evidence for the oxidative conversion of tBHQ and DtBHQ to BQs, a ^{1}H NMR analysis of tBHQ and DtBHQ upon incubation with Cu^{2+} was performed without purification of BQ. The oxidation of tBHQ and DtBHQ by Cu^{2+} was performed in the presence of bathcuproine to remove Cu^+, which catalyzes the reversed reductive reaction. The oxidized products of tBHQ and DtBHQ by Cu^{2+} were extracted in chloroform-*d* to allow measurement of the ^{1}H NMR spectra (Figure 3A). When

tBHQ and DtBHQ were treated with Cu^{2+}, the spectra assigned to *tert*-butyl-BQ and 2,5-di-*tert*-butyl-BQ, respectively, were observed. Although hydroquinone (1,4-dihydroxybenzene) was reported to be completely oxidized into BQ by equimolar quantities of Cu^{2+} within 1 min (*28*), significant amounts of tBHQ and DtBHQ remained in the same condition.

On the other hand, during the oxidation process, Cu^{+} is generated by the reduction of Cu^{2+} (*37*). It is generally accepted that the Cu^{+} ion can produce O_2^- through its reaction with oxygen. Hence, to investigate whether O_2^- is produced in the redox cycle of tBHQ and DtBHQ, we measured the O_2^- generation using a cytochrome *c* reduction method. We estimated the amount of O_2^- generation from the difference in the cytochrome *c* reduction with or without SOD. As shown in Figure 3B, tBHQ and DtBHQ produced a significant amount of O_2^-. These results suggested that DtBHQ has a propensity for redox cycling similar to tBHQ.

Electrophilic Properties of Oxidized tBHQ andDtBHQ. It is suggested that the subsequent oxidation of tBHQ and DtBHQ generates BQs, which are capable of reacting with nucleophiles such as GSH. Hence, we incubated tBHQ and DtBHQ with GSH and evaluated their electrophilicity by measuring the reactivity with GSH. As shown in Figure 4A, the reduced GSH level was observed after the addition of tBHQ but not DtBHQ. This effect of tBHQ is dose-dependent and was counteracted by the coexistence of 2-mercaptethanol (Figure 4B). As the number of *tert*-butyl moieties increased, a lower amount of reduced GSH was consumed. Similar results were obtained by an alternative procedure using a reversed-phase HPLC with an electrochemical detector (HPLC-ECD technique), which can detect a free thiol group (data not shown).

We then examined the formation of the tBHQ-GSH adduct within the cells. To this end, RL34 cells in the monolayer culture were exposed to 10 μM tBHQ, and the tBHQ-GSH conjugate was analyzed by the HPLC-ECD technique. As shown in Figure 4C, tBHQ was incorporated very rapidly into the cells, reaching 120 nM at 30 min. The cellular concentration began to gradually decrease thereafter. Accompanied by the incorporation of tBHQ, two tBHQ-GSH adducts (Figure 4D), 5-glutathion-*S*-yl-tBHQ (5-GS-tBHQ) and 6-glutathion-*S*-yl-tBHQ (6-GS-tBHQ), were detected in the tBHQ-treated RL 34 cells (Figure 4E). Quantification of the GSH-conjugates showed that the major one was 5-GS-tBHQ (2.5% of intracellular tBHQ concentration) with a lower amount of 6-GS-tBHQ (0.5% of intracellular tBHQ concentration), both of which were quickly formed within the cells (Figure 4F). 3,6-Bis-glutathion-*S*-yl-tBHQ, detected as a metabolite of tBHQ in rat bile (*38*), was not detected in RL34 cells. In total, the conjugated GSH accounted for 3% of the tBHQ concentration. These data taken together indicated that DtBHQ possesses a redox-active character but no detectable electrophilic ability, whereas tBHQ has both properties.

Effect of tBHQ and DtBHQ on GST Induction in RL34 Cells. In a previous study, we found that the activity toward CDNB, a general GST substrate, in inducer-sensitive RL 34 cells was most potently induced by 1,4-diphenols including tBHQ among the phenolic antioxidant derivatives (*8*). These data were consistent with that reported previously by Prochaska et al. (*39*) that the induction of NQO in murine

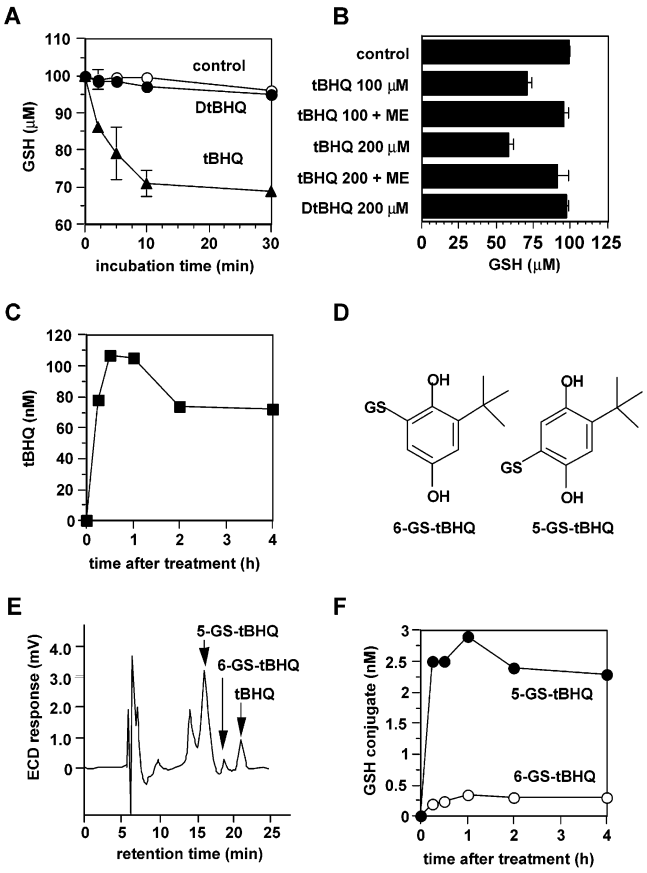


FIGURE 4: Differences in electrophilic properties of tBHQ and DtBHQ. (A) GSH consumption by tBHQ and DtBHQ. Reactions were performed with 100 μM tBHQ (closed triangle) or DtBHQ (closed circle) with 100 μM GSH in 1 mL of 100 mM phosphate buffer (pH 7.4). The amount of residual GSH was estimated spectrophotometrically using commercial kit GSH-400. (B) Counteracting effect of 2-ME (1 mM) on GSH conjugates formation. (C) Detection of intracellularly accumulated tBHQ and tBHQ-GSH conjugates by HPLC-ECD. (D) Chemical structures of tBHQ-GSH conjugates. (E) Quantification of intracellularly accumulated tBHQ. (F) Quantification of intracellularly accumulated GSH-tBHQ conjugates.

Hepa1c1c7 hepatoma cells depends on the oxidation−reduction lability. To further investigate which properties, either redox cycling-active or electrophilic, are involved in the GST induction by phenolic antioxidants, we examined the GST-inducing ability of DtBHQ as compared with tBHQ. It should be noted that the modifying effect of DtBHQ on the phase II enzyme expression has not yet been determined, although DtBHQ, among the hydroquinone derivatives, is the only exception to protect against benzo[*a*]pyrene-induced neoplasia of the forestomach in ICR mice (*39*). Figure 5A shows the modifying effects of 10, 20, 50, and 100 μM concentrations of tBHQ and DtBHQ in RL34 cells on the GST activities toward EA, a specific substrate of Class pi GSTs as well as CDNB. The mean basal specific activities of the cytosolic GSTs in RL34 cells were 16.8 ± 2.1 and 8.3 ± 0.7 ($\times 10^{-3}$) units per mg of protein, respectively. tBHQ potently enhanced the activities toward CDNB in a dose-dependent manner. These results suggested that half of the GST induction by tBHQ was accounted for the induction of Class pi GST isozymes. On the other hand, the inducibility of DtBHQ, significantly generating ROS, was not efficient even at a concentration of 100 μM. In addition,

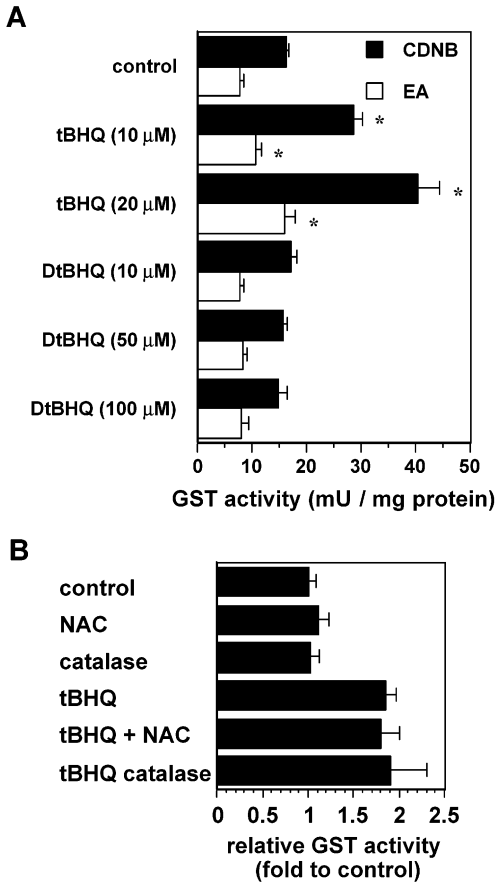


FIGURE 5: Induction of GST activity by tBHQ and DtBHQ in rat liver epithelial RL34 cells. (A) Dose-dependent effect of tBHQ and DtBHQ on cellular GST activity. Cells post-confluency were exposed to the test compounds in the medium containing 5% fetal bovine serum. Cells were exposed to the test compounds for 24 h. The cellular GST activity was evaluated according to the method of Habig et al. (*29*). (B) The effect of antioxidants on tBHQ-induced GST induction. RL34 cells were pretreated with vehicle (PBS), NAC (2.5 mM), and catalase (100 unit/mL) for 2 h and then treated with tBHQ (10 μM) for 24 h.

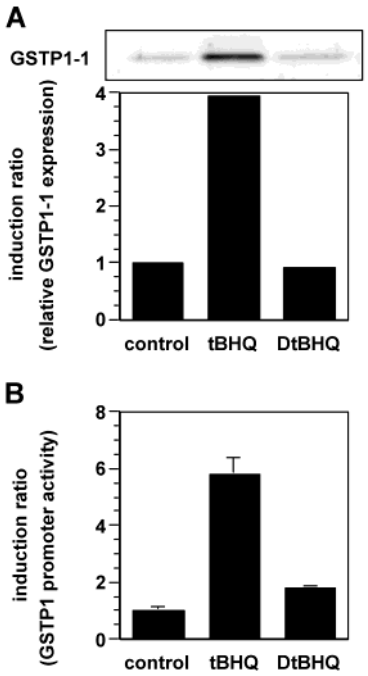


FIGURE 6: tBHQ but not DtBHQ induces GSTP1-1 protein expression and transcription of an GSTP1 gene 5′-flanking region-CAT reporter gene. (A) Effect of tBHQ and DtBHQ on GSTP1 protein. Cells post-confluency were exposed to tBHQ (10 μM) and DtBHQ (100 μM) in the medium containing 5% fetal bovine serum for 24 h. GSTP1-1 level was examined by an immunoblot analysis. (B) Stimulation of promoter activity of *GSTP1* by tBHQ and DtBHQ. The bacterial CAT reporter gene harboring the 5′-flanking region (−2.9 kb to + 59 bp) of the *GSTP1* gene was transfected into RL34 cells and treated with 10 μM tBHQ or 100 μM DtBHQ.

the tBHQ-induced enhancement of Class pi GST isozymes activity, measured by EA conversion, was not counteracted by the antioxidant treatment (Figure 5B). These results led us to the hypothesis that ROS produced by redox cycling can be ruled out in the major mechanism of the *GSTP1* induction by tBHQ.

tBHQ Is a Potent Inducer of GSTP1, and GPEI Is the tBHQ Response Element. To further confirm whether tBHQ or DtBHQ influences the expression of the pi Class GST protein, the main GST isozyme in RL34 cells, an immunoblot analysis was carried out using the GSTP1-1-specific antibody. Figure 6A demonstrates a significant increase in the level of GSTP1-1 by the treatment with tBHQ but not with DtBHQ at a concentration of 25 μM. No inducing potency of DtBHQ to induce the GSTP1-1 protein was also observed up to a concentration of 100 μM (data not shown). Next, to determine whether tBHQ or DtBHQ stimulates the promoter activity of the *GSTP1* gene, we examined the effect of tBHQ or DtBHQ on the transient expression of the bacterial CAT reporter gene harboring the 5′-flanking region (−2.9 kb to +59 bp) of the *GSTP1* gene in RL34 cells. As shown in Figure 6B, ECAT containing the 5′-flanking region showed a low but detectable CAT activity, and tBHQ strongly stimulated it by about 6-fold. On the other hand, the stimulating effect of DtBHQ on the promoter activity was also detectable but much weaker (2-fold) than that of tBHQ. This indicates that the region contains an element responsible for tBHQ but not DtBHQ. Because DtBHQ does not show any reactivity with thiols but has the ability to generate ROS similar to tBHQ, DtBHQ slightly induces the *GSTP1* expression possibly through a redox cycling-dependent mechanism. On the contrary, an electrophilic property may play a major role in tBHQ-stimulated *GSTP1* induction.

We have previously reported that the 5′-flanking region of the *GSTP1* gene contains an element responsible for benzyl isothiocyanate as previously reported (*9*). To identify the element responsible for the tBHQ-stimulated promoter activity, we utilized a series of constructs including the 5′-flanking region of the *GSTP1* gene, namely ECAT and its deletion mutants (Figure 7B). As shown in Figure 7B, the 1CAT had completely lost the basal and tBHQ responsiveness, indicating that the tBHQ element of the *GSTP1* gene is located between −2.9 to −2.2 kb upstream from the transcription start site. Deletion to −1.4 kb (2CAT) and −0.8 kb (3CAT) showed only slight changes in the CAT activity, while further deletion to −0.14 kb (4CAT) resulted in a significant recovery of both the basal and the tBHQ-induced CAT activities, suggesting that some element suppressing and/or enhancing the transcription activity is also located between −0.4 and −0.14 kb and/or between −0.14 and +59 kb, respectively. It should be noted that a silencer element has previously been identified in the region between −0.4 and −0.14 kb (*40*). There is a strong enhancer element,

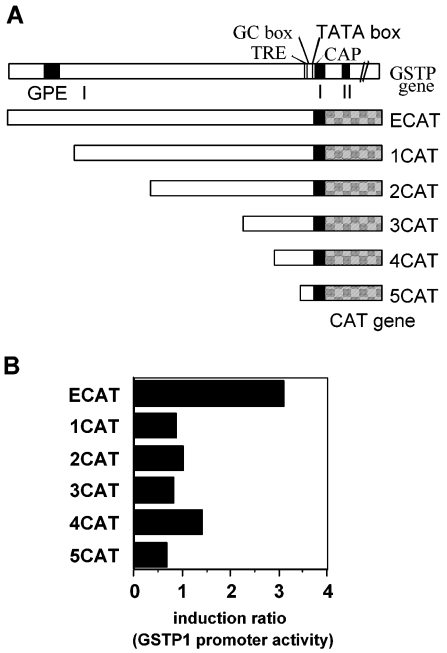


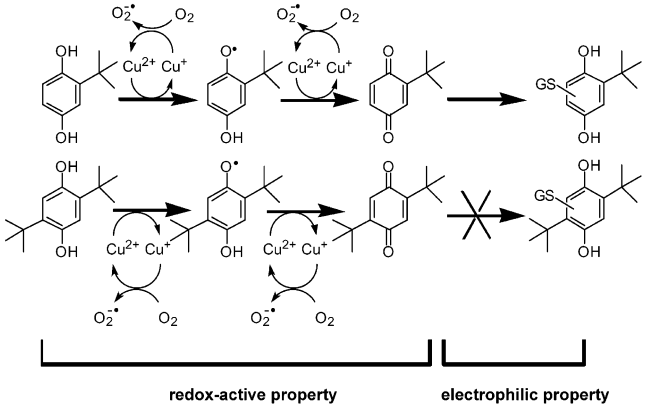
FIGURE 7: Determination of the DNA element required for the induction *GSTP1* expression by tBHQ. (A) 5′-deletion constructs are schematically indicated. (B) 5 μg of CAT constructs was transfected into RL34 cells and treated with 10 μM tBHQ.

GPEI, in the restricted region between −2.9 and −2.2 kb (*41*). A further experiment using the construct that has the minimum *GSTP1* promoter connected to the CAT coding region indicated that tBHQ stimulated the CAT activity by about 2- to ∼2.5-fold in the cells transfected with the core GPEI-containing construct, while mutation of either of the TRE-like elements strongly reduced the tBHQ-induced promoter activity (data not shown). These data strongly indicated that the GPEI represents the tBHQ reponsible element and that both TRE-like elements in this element are essential for this stimulation of the promoter activity.

DISCUSSION

Diverse chemical compounds, including Michael reaction acceptors, quinones, catechols, peroxides, isothiocyanates, mercaptans, heavy metals, and planar polycyclic aromatic hydrocarbons, induce the expression of the phase II detoxifying enzymes. The induction of several genes in the phase II enzyme battery is mediated through ARE or its functional equivalent, an electrophile response element, EpRE, found in the mouse GST Ya gene (*42*). It has been hypothesized that these inducing agents share in common the ability to generate reactive oxygen intermediates directly or via redox cycling that result in the activation of transcription factors, possibly through their alterations of the redox status. While the ROS-dependent mechanism is favored by some researchers, Talalay and colleagues pointed out that most of the phase II inducers are capable of generating electrophilic intermediates and have the potential to react with nucleophilic residues of proteins including sulfhydryl groups (*16*). Therefore, they have speculated that a better designation for these responsive elements would be EpREs to reflect the nature of the active intermediates. Recently, the correlation between the phase II drug metabolizing ability and electroplilic feature has clearly been demonstrated by structure−activity relationship

Scheme 1: Summary of Redox Cycling and Electrophilic Potentials of tBHQ and DtBHQ



studies of the naturally occurring and synthetic chemicals (*43*).

Although the antioxidant activity of tBHQ has been well-studied by many researchers, the fate of tBHQ after exhibiting a radical scavenging ability is not fully understood. We demonstrated here that DtBHQ showed a significant scavenging activity toward a stable water-soluble radical, DPPH (Figure 2A), and its activity is similar to that of tBHQ. It should be noted that, not only during the auto-oxidation process but also during the radical scavenging reaction, tBHQ is mainly converted to *tert*-butylbenzoquinone (*44*), which is easy to be substituted by nucleophiles by Michael addition type reaction. It is possible that DtBHQ can be oxidized to convert to the electrophilic BQ form. No correlation between the potential to induce the *GSTP1* expression and the antioxidant efficacy of the HQ derivatives indicated that the antioxidative reaction of the hydroquinone derivatives could be ruled out in the molecular mechanism. Therefore, it is suggested that the metabolic product(s) of tBHQ should be taken into account in the mechanism of the GST induction. In addition, DtBHQ exhibits not only radical scavenging activity in vitro (Figure 2A) but also inhibitory effects against intracellular oxidative stress induced by a GSH-depleting agent, DEM (Figure 2B). It is thus likely that the oxidative conversion of DtBHQ to the BQ form may occur within cells, as shown in Scheme 1.

The ROS generating ability of tBHQ has been demonstrated to play important roles in some biological or chemical systems (i.e., cytotoxicity), oxidative DNA damage, and activation of transcriptional factors including AP-1 and NF-κB. As described above, the hydroxyl radical has been suggested to be a critical intermediate of the tBHQ-triggered ARE-dependent gene expression including *rGSTA1* (*17*). The redox cycling between tBHQ (fully reduced), the semiquinone radical (one-electron oxidation product), and *tert*-butylbenzoquinone (fully oxidized by two electrons) is concomitant with the formation of O_2^-, a one-electron reduction product of molecular oxygen (Scheme 1). Indeed, DtBHQ generated O_2^- in an amount comparable to tBHQ (Figure 3B) and thus induces oxidative cleavage of DNA as previously reported (*45*). We thus concluded that DtBHQ possesses a redox-active character in a manner similar to tBHQ. However, there is no correlation between the ROS generation ability and the GST induction potential of tBHQ and DtBHQ.

We also demonstrated in the present study that the significant yield of BQs of tBHQ and DtBHQ was obtained by the Cu^{2+}-mediated oxidation in vitro (Figure 3A). On the other hand, the in vitro experiments clearly showed that DtBHQ has a poor reactivity with reduced GSH, while tBHQ was quickly and stoichiometrically consumed upon incubation with GSH (Figure 4A). The structural difference between DtBHQ and tBHQ is only a bulky *tert*-butyl moiety, and DtBHQ has two *tert*-butyl groups on both sides against the axis linking the two hydroxyl groups of the hydroquinone moiety (Figure 1). The lower ability of DtBHQ to conjugate with GSH was supported by the in vivo metabolic study of tBHQ that indicated that the Micheal addition of the GSH thiol group to tBHQ occurs easily on the opposite side of the *tert*-butyl moiety than on the same side (*38*). These results clearly indicated that interference of the GSH conjugation by the introduction of the *tert*-butyl moiety may be due to its steric hindrance rather than to its electronic characteristics, although the *tert*-butyl moiety is an electron-donating group and reduces the reaction rate of the Michael addition to the m- or p-position. This assumption was also supported by the quantum mechanical computer calculation study that the energy of the lowest unoccupied molecular orbital (LUMO) of DtBHQ is similar to that of tBHQ (unpublished data). The topic of the present study is that DtBHQ, which has a propensity for redox cycling similar to that mentioned above but is hardly reacting with GSH probably because of steric hindrance of the bulky *tert*-butyl moieties, has less ability for GST induction. Thus, we concluded that an electrophilic quinone oxidation product that reacts with intracellular nucleophiles including protein thiol or GSH is the pivotal inducer of the *GSTP1* gene expression.

It is believed that activation of the phase II enzyme genes is regulated by signal transducing kinase cascades (*46*). Yu et al. (*47*) have recently identified extracellular signal-regulated protein kinase (ERK) pathway to be involved in the ARE-mediated induction of phase II enzymes by tBHQ and an isothiocyanate compound. They have also shown that the induction involves the direct activation of Raf-1 (a MAPK kinase kinase), which phosphorylates and activates MEK (a MAPK kinase). Our preliminary experiment has also demonstrated that tBHQ indeed stimulates the phosphorylation of ERK1 and *c*-Jun *N*-terminal kinase (Naito, Y., Nakamura, Y., and Uchida, K., unpublished data). It is therefore likely that activation of Raf-1 followed by stimulation of the ERK kinase pathway is involved in the induction of *GSTP1* by tBHQ. More recently, Yu et al. have also shown that the p38 MAP kinase negatively regulates the induction of the phase II drug-metabolizing enzymes (*48*). Interfering with the p38 kinase pathway by overexpression of a dominant-negative mutant of p38 or mitogen actived protein kinase kinase 3 (MKK3), an immediate upstream regulator of p38, potentiated the activation of the ARE reporter gene by tBHQ, whereas the wild types of p38 and MKK3 diminished such activation. These results suggested that the coordinate modulation of MAP kinase cascades may be critical to the regulation of the phase II enzyme genes through the ARE induced by various chemopreventive agents (*49*).

We showed here that tBHQ stimulated the promoter activity of the 5′-flanking region of the *GSTP1* gene (Figure 6B) and then induced GST mRNA and protein in RL34 cells (Figure 6A). It appeared that this stimulation required a specific region containing GPEI that specifically responded to tBHQ activation (Figures 6B and 7). It has been shown that the enhancer of the *GSTP1* expression is regulated by multiple factors, including AP-1, which is known to be a heterodimer composed of the products of the *jun* and *fos* oncogenes (*50*). *c*-Jun is a member of a multiprotein family that has been implicated in a number of signal transduction pathways associated with cellular growth, differentiation, neuronal excitation, and cellular stress (*51*−*54*). It may be likely that *c*-Jun functions as an important component that activates GPEI followed by the expression of *GSTP1*. On the other hand, another candidate of the trans-acting factor-(s) for the induction of GST and other phase II enzymes has been recently identified. Venugopal and Jaiswal (*55*) have reported that the transcription factor NF-E2-related factor 2 (Nrf2) positively regulates the ARE-mediated expression of the phase II detoxification enzyme genes. Itoh et al. (*56*) have also shown by gene-targeted disruption in mice that Nrf2 is a general regulator of the phase II enzyme genes in response to electrophiles and ROS. The general regulatory mechanism underlying the electrophile counterattack response has been demonstrated, in which electrophilic agents alter the interaction of Nrf2 with its repressor protein (Keap1), thereby liberating Nrf2 activity from repression by Keap1, culminating in the induction of the phase II enzyme genes and antioxidative stress protein genes via AREs (*57*). Keap 1 contains 25 cysteine residues, nine of which are expected to have highly reactive sulfhydryl groups (*58*). More recently, Dinkova-Kostova et al. reported direct evidence that sulfydryl groups of Keap1, covalently modified by nucleophilic agents, act as the sensors regulating the induction of the phase II enzymes (*59*). Therefore, the Keap1−Nrf2 complex is the most plausible candidate for the cytoplasmic sensor system that recognizes inducers including tBHQ. DtBHQ can be used as a nonelectrophile control for the identification of sensor protein. Although the participation of Nrf2 in the mechanism of the *GSTP1* induction by tBHQ is not fully elucidated, further studies of the signaling pathways between electrophilic attachment to a plausible target molecule and GST gene expression are essential to advance our understanding of the efficacy and safety of phase II enzyme inducers as therapeutic and preventive agents.

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BI0340090