Butylated Hydroxyanisole and Its Metabolite tert-Butylhydroquinone Differentially Regulate Mitogen-Activated Protein Kinases

THE ROLE OF OXIDATIVE STRESS IN THE ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES BY PHENOLIC ANTIOXIDANTS*

(Rceived for publication, June 17, 1997, and in revised form, August 22, 1997)

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Phenolic antioxidant butylated hydroxyanisole (BHA) is a commonly used food preservative with broad biological activities, including protection against acute toxicity of chemicals, modulation of macromolecule synthesis and immune response, induction of phase II detoxifying enzymes, and especially its potential tumor-promoting activities. Understanding the molecular basis underlying these diverse biological actions of BHA is of great importance. Here we demonstrate that BHA is capable of activating distinct mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase 2 (ERK2), and c-Jun N-terminal kinase 1 (JNK1). Activation of ERK2 by BHA was rapid and transient, whereas the JNK1 activation was relatively delayed and persistent. A major metabolite of BHA, tert-butylhydroquinone (tBHQ), also activated ERK2 but weakly stimulated JNK1 activity. Furthermore, tBHQ activation of ERK2 was late and prolonged, showing a kinetics different from that induced by BHA. ERK2 activation by both compounds required the involvement of an upstream signaling kinase MAPK/ERK kinase (MEK), as evidenced by the inhibitory effect of a MEK inhibitor, PD98059. Pretreatment with N-acetyl-L-cysteine, glutathione, or vitamin E attenuated ERK2 but not JNK1 activation by BHA and tBHQ. Modulation of intracellular H₂O₂ levels by direct addition of catalase or pretreatment with a catalase inhibitor, aminotriazole, also affected BHA- and tBHQ-stimulated ERK2 activity but not JNK1, indicating the involvement of oxidative stress in the ERK2 activation by these two compounds. However, we did not observe any generation of H₂O₂ after exposure of cells to BHA or tBHQ using a H₂O₂-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate. Instead, BHA and tBHQ substantially reduced the amount of intracellular H₂O₂. Furthermore, BHA and tBHQ activation of ERK2 was strongly inhibited by ascorbic acid and a peroxidase inhibitor, sodium azide, suggesting the potential role of phenoxyl radicals and/or their derivatives. Taken together, our results indicate that (i) BHA and its metabolite tBHQ differentially regulate MAPK pathways, and (ii) oxidative stress due to the generation of reactive intermediates, possibly phenoxyl radicals but not H₂O₂, is responsible for the ERK2 activation by BHA and tBHQ, whereas the JNK1 activation may require a distinct yet unknown mechanism.

Butylated hydroxyanisole (BHA), a synthetic phenolic antioxidant due to its chain-breaking action during the autooxidation of lipid, is widely used as a food preservative, probably ubiquitous in almost all food additives (1, 2). In addition to its action as an inhibitor of lipid peroxidation, this compound exhibits a wide range of biological activities. BHA protects animals against radiation and the acute toxicity of various xenobiotics and mutagens (3, 4). Dietary administration of BHA also leads to the protection against various carcinogens, presumably through the induction of many phase II detoxifying enzymes such as epoxide hydrolases (5, 6), glutathione S-transferases (7), and glucuronosyltransferases (8), as well as through the inhibition of cytochrome P-450 monooxygenase (9). On the other hand, a growing body of evidence indicates that BHA may also be a tumor initiator or a tumor promoter in some tissues of animals. For example, BHA induced papilloma and carcinoma formation in the forestomachs of rats, mice, and hamsters when fed continuously at high concentrations (10, 11). Chronic dietary administration of BHA also enhances the development of preneoplastic and neoplastic lesions in the rat kidney and urinary bladder (12, 13). Furthermore, BHA appears to have initiating activity in two-stage carcinogenesis assay and in two-stage transformation of BALB/c3T3 cells (14). Most notably, BHA induced proliferative effects not only in rodent forestomachs but also in the esophagus of pigs and primates (15, 16). Thus, this well known antioxidant exerts opposing biological effects. Although both anti-carcinogenic and carcinogenic effects of BHA are well described, the precise mechanisms of how these effects are achieved remain obscure but probably are dose- and/or tissue-dependent.

Studies on the metabolism of BHA revealed that several metabolic pathways may exist, including dimerization, conjugation, and O-demethylation (17). One of the major metabolites of BHA, as shown in dogs (18), rats, and man (19), and in rat liver microsomes (20), is the demethylated product, tert-butylhydroquinone (tBHQ), which also exhibits anti-carcinogenic

* This work was supported by National Institutes of Health Grants R01-GM49172 and R01-ES06887 (to A.-N. T.) and R01-GM49875 and R01-ES06887 (to T.-H. T). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: BHA, butylated hydroxyanisole; tBHQ, tert-butylhydroquinone; MAPK, mitogen-activated protein kinase; ERK2, extracellular signal-regulated protein kinase 2; JNK1, c-Jun N-terminal kinase 1; NAC, N-acetyl-L-cysteine; V-E, vitamin E; ROS, reactive oxygen species; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; MEK, MAP kinase kinase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; MBP, myelin basic protein.
properties in some animal models of cancer in a manner similar to that described for BHA. This includes modulation of the enzyme systems responsible for metabolic activation or deactivation of chemical carcinogens (21). Thus, metabolic formation of tBHQ is thought to contribute to the anti-carcinogenic activities of BHA. However, tBHQ is also shown to be a carcinogen in many animal tissues. Although the reason why tBHQ is carcinogenic remains unknown, the oxidation of tBHQ to its corresponding quinone, tert-butylyquinone, accompanied by the generation of reactive oxygen species (ROS), presumes to play an important role (22–24). Since BHA did not show genotoxic activity in most tests for mutagenicity (25, 26), tBHQ-mediated generations of tert-butylyquinone and ROS, which are known to cause DNA damage, is believed to be responsible for BHA-induced carcinogenesis. However, several studies suggested that such carcinogenic effects of BHA are tBHQ- or tert-butylyquinone-independent and are epigenetic (19, 23, 27). While the relevance of metabolism of BHA for its biological action has been the focus of many studies over the years, the signal transduction pathways that control the cellular responses to BHA and its metabolites have not been elucidated.

Mitogen-activated protein kinases (MAPKs), characterized as proline-directed serine/threonine kinases (28), are important cellular signaling components that convert various extracellular signals into intracellular responses through serial phosphorylation cascades (29). At the present time, three distinct but parallel MAPK cascades have been identified in mammalian cells (30). Each consists of a module of three kinases as follows: a MAPK kinase kinase, which phosphorylates and activates a MAPK kinase, which, in turn, phosphorylates and activates a MAPK. The best characterized MAPK pathway is a Ras-dependent activation of extracellular signal-regulated protein kinases (ERKs) in response to growth factors. In this pathway, tyrosine-phosphorylated transmembrane receptors associate with the SH2 domain of the adapter protein Grb2 (31) and target nucleotide exchange factor SOS to the membrane-bound small G-protein Ras (32). Activated Ras recruits Raf-1 (a MAPK kinase kinase) to the membrane, leading the activation of Raf-1 (33). Once activated, Raf-1 can phosphorylate and activate a dual specificity kinase MEK (a MAPK kinase), which, in turn, activates ERK (a MAPK). In addition to tyrosine kinase receptors, certain G-protein-coupled receptors and protein kinase C are also capable of activating ERK cascade components as demonstrated by the inhibitory effects of a MEK-specific inhibitor PD98059. Further studies using different free radical scavengers and flow cytometry revealed that formation of non-oxygen free radicals, possibly phenoxyl free radicals, from parent molecules of BHA and tBHQ, represents a major mechanism for ERK2 activation but not for JNK1.

**MATERIALS AND METHODS**

**Cell Culture, Antibodies, DNA Plasmons, and Chemicals—Human HeLa cells and Hep G2 cells, obtained from American Type Culture Collection (Rockville, MD) were maintained as monolayer cultures in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 g/liter sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were normally starved overnight in serum-free medium without phenol red before treatment. Polyclonal antibody to ERK2 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-JNK1 antiserum (Ab101) was described previously (38, 43). GST-c-Jun(1–79) expressing plasmid was kindly provided by Dr. M. Karin (University of California, San Diego, CA), and the GST-c-Jun fusion protein was purified from Escherichia coli lysates with the aid of glutathione-Sepharose beads (Pharmacia Biotech Inc.). PD95089, a specific inhibitor of MEK, was obtained from New England Biolabs Inc. Fluorescence dyes 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Molecular Probes, Inc. (Eugene, OR). Myelin basic protein, catalase, dismutase, NAC, reduced glutathione (GSH), α-tocopherol (vitamin E; V-E), ascorbic acid (vitamin C), 3-aminoazale, hydrogen peroxide (H₂O₂), and BHA were obtained from Sigma. tBHQ was purchased from Aldrich. [γ-32P]ATP (3,000 Ci/mmol) was obtained from NEN Life Science Products.

**Immunoprecipitation and ERK2 Kinase Activity Assay—After treatment with BHA or tBHQ (both agents were dissolved in ethanol, the final concentration of ethanol in the culture medium was controlled to less than 0.1%), monolayer cells, grown in a 10-cm diameter plate, were washed with ice-cold phosphate-buffered saline (PBS) and harvested in 700 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM inorganic sodium pyrophosphate, 50 mM sodium fluoride, 100 mM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM zinc chloride, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. The lysate was homogenized by passing through a 23-gauge needle three times. After 15 min on ice, the homogenate was centrifuged at a high speed for 10 min at 4 °C, and the supernatant was transferred into a new tube. ERK activity was determined by an immunocomplex kinase assay as described previously (43). Briefly, equal amounts of protein, as measured by Bio-Rad protein assay (Bio-Rad, CA), were incubated with rabbit anti-ERK2 polyclonal antibody and protein A-Sepharose beads for 2 h at 4°C. The immunoprecipitate was washed and resuspended for 1 min and washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES (pH 7.9), 10 mM magnesium chloride, 2 mM manganese chloride, 0.1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate). Kinase reaction was initiated by resuspending the immunoprecipitate in 30 μl of kinase assay buffer containing 10 μg of myelin basic protein, 2 μl of 10 mM [γ-32P]ATP, and 20 μl ATP. After incubation for 15 min at 30 °C, the reaction was terminated by adding 10 μl of 4 × Laemmli’s buffer and heating to 95 °C for 5 min; samples were resolved in 13.5% SDS-polyacrylamide gel electrophoresis. Gel was stained with Coomassie Blue and then washed overnight and dried. The phosphorylation of myelin basic protein was visualized by autoradiography and quantified with a PhosphorImager (AMBI, San Diego, CA).

**JNK1 Activity Assay—**JNK1 activity was assayed according to the procedures described above for ERK2 assay, with the following changes. After immunoprecipitation with rabbit anti-JNK1 antisemur, JNK1 activity was detected using 10 μg of GST-c-Jun(1–79) fusion protein as substrate. Kinase reaction was performed at 30 °C for 30 min. The phosphorylated product was resolved in 10% SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography and phosphorimage.

**Fluorescent Measurement of Intracellular Peroxides—**Confluent, serum-starved HeLa cells and Hep G2 cells were treated with different agents and then incubated with 5 μM DCF-DA (dissolved in MeSO) for an additional 30 min at 37 °C. After chilling on ice, cells were washed with ice-cold PBS, scraped from the plate, and resuspended at 5 × 10⁶ cells/ml in PBS containing 2% fetal bovine serum and 5 μM DCF-DA.
The fluorescence intensities of DCF-DA of more than 10,000 viable cells from each sample were analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 485 and 525 nm, respectively. Prior to data collection, propidium iodide was added to the sample for gating out dead cells.

Cell Viability Assay—Cells were plated at a density of $5 \times 10^4$ cells/well into 96-well plates, with each well containing 100 $\mu$L of medium. After overnight recovery, cells were treated with a series of concentrations of BHA or tBHQ. Drugs were removed after 4 h of treatment, and cells were cultured in the fresh medium for an additional 24 h and then assayed for viability using CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 20 $\mu$L of combined solution of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, and an electron coupling reagent phenazinemethosulfate was added to each well. After incubation for 1 h at 37 °C in a humidified 5% CO$_2$ atmosphere, the absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay plate reader.

RESULTS

**BHA Strongly Activates Both ERK2 and JNK1 in HeLa Cells and Hep G2 Cells—**HeLa cells, which are derived from a human cervical squamous cell carcinoma, are used as a model system for the studies of MAPK in response to various factors. Hep G2, a human hepatoma cell line, has been widely used to study the role of metabolism of different compounds, because of the extensive metabolic capacity of this cell line. In the present experiment, we have examined the effects of BHA on ERK2 and JNK1 activities in these cell lines. As illustrated in Fig. 1A, BHA strongly stimulated ERK2 activity in a dose-dependent fashion in both cell lines after 25 min of treatment. In HeLa cells, ERK2 activation occurred when the concentration of BHA reached 250 $\mu$M. In Hep G2 cells, the stimulated ERK2 activity, however, appeared at a much lower concentration, around 50 $\mu$M. The maximal induction of ERK2 activity was observed at 500 $\mu$M in HeLa cells and 250 $\mu$M in Hep G2 cells. Further increasing concentrations of BHA to 750 $\mu$M dramatically reduced ERK2 activity to control level in Hep G2 cells or to the level slightly higher than control in HeLa cells. Similar to ERK2, JNK1 was also strongly activated in both cell lines after 90 min of treatment with BHA (Fig. 1B). Interestingly, activation of both kinases followed a similar dose-response pattern.

Based on the dose-response data, we next studied the kinetics of ERK2 and JNK1 activation by BHA. In HeLa cells, 500 $\mu$M BHA activated ERK2 in a time-dependent fashion (Fig. 2A). The induced ERK2 activity reached maximum within 15 min and disappeared after 60 min of treatment with BHA. Similar to that observed in HeLa cells, activation of ERK2 in Hep G2 cells by 250 $\mu$M BHA peaked at 15 min. Afterward, a rapid decrease in ERK2 activity ensued. Unlike ERK2 activation, JNK1 activation was delayed and seen at 15 min in HeLa cells or at 30 min in Hep G2 cells, instead of 5 min in ERK2 activation (Fig. 2B). Furthermore, after peaking at 2 h, JNK1 activity gradually declined to control levels in both cell lines, showing a sustained activation pattern. Different kinetics of ERK2 and JNK1 activation suggest that these two kinases are differentially regulated in response to BHA. When Western blotting analysis was performed with anti-ERK2 or anti-JNK1 antibodies, no changes in the protein levels of ERK2 or JNK1 occurred throughout the kinetic studies, indicating that the activation of ERK2 or JNK1 resulted from the phosphorylation of pre-existing kinase molecules instead of the de novo protein synthesis (data not shown).

**tBHQ Activates ERK2 in Hep G2 Cells but Not in HeLa Cells and Is Also a Weak Inducer of JNK1—**tBHQ is a major metabolite of BHA, as demonstrated in many animal species as well as in human (18, 19). A number of previous studies implicated that this compound may mediate many biological activities of BHA, including anti-carcinogenic and carcinogenic effects.
different concentrations of tBHQ for 30 min, as shown in Fig. 3B. No significant induction of ERK2 activity was observed, regardless of the dose and time of the treatments. However, under similar conditions, BHA strongly activated ERK2, with a maximum of more than 18-fold induction, as seen in Fig. 2A.

Differential regulation of ERK2 signaling pathway by BHA and tBHQ prompted us to examine whether these two compounds also differentially regulate another member of the MAPK family, JNK. To do so, we first studied the time course of JNK1 activation by tBHQ. Both HeLa cells and Hep G2 cells were treated with 500 μM tBHQ for different periods. As shown in Fig. 3C, JNK1 activity was weakly stimulated by tBHQ, with a maximum of 2.2-fold induction in HeLa cells and 2.7-fold induction in Hep G2 cells. To exclude the possibility that JNK1 activity was measured at an unfavorable concentration, we then investigated the dose response of JNK1 activation by tBHQ. Similar to the results obtained in the kinetic studies (Fig. 3B), no substantial JNK1 activation was observed (data not shown). Therefore, unlike BHA, tBHQ was a weak inducer of JNK1 activity.

**Activation of ERK2 by BHA and tBHQ Requires the Involvement of an Upstream Signaling Kinase MEK**—Activation of ERK2 is a consequence of serial phosphorylation of upstream kinases (28). Among these kinases is MEK (a MAPK kinase), which activates ERK2 in response to various stimuli, such as growth factors (45) and oxidative stress (46), by phosphorylation of ERK2 on threonine and tyrosine residues in a consensus TGY motif. To determine whether BHA and tBHQ utilize a MEK-dependent pathway, we took advantage of the recently identified MEK inhibitor, PD98059 (47, 48). Since both BHA and tBHQ activated ERK2 in Hep G2 cells, we conducted the experiment in this cell line. In mock-pretreated Hep G2 cells (0.1% Me2SO), both BHA (250 μM, 15 min) and tBHQ (500 μM, 30 min) strongly activated ERK2 (Fig. 4). Pretreatment of the cells with 25 or 50 μM PD98059 for 60 min abrogated ERK2 activation by both BHA and tBHQ. PD98059 at 50 μM alone slightly reduced ERK2 basal activity. These data demonstrated that ERK2 activation by BHA and tBHQ requires upstream signaling components and is MEK-dependent. However, JNK1 activation by these two compounds was unaffected by pretreatment with PD98059 (data not shown), consistent with the
Catalase is a highly reactive enzyme and breaks down H$_2$O$_2$ to NAC, and involvement of reactive free radical species. As shown in Fig. 3, the inhibitory effects of these antioxidants would implicate the involvement of these oxidative metabolites using several potent antioxidants, glutathione (GSH), N-acetyl-l-cysteine (NAC), and α-tocophenol (V-E). GSH, the most abundant intracellular thiol compound in mammalian cells, is a scavenger of many free radicals and a substrate of glutathione peroxidase which degrades intracellular hydrogen peroxide (H$_2$O$_2$). NAC is a precursor of GSH, and it can also directly scavenge many different types of reactive free radicals, because of its low reduction potential. Vitamin E is a lipid soluble antioxidant that is also highly reactive toward lipid-soluble radicals. Thus, the inhibitory effects of these antioxidants would implicate the involvement of reactive free radical species. As shown in Fig. 5A, the BHA-stimulated ERK2 activity was inhibited by 95 ± 5, 88 ± 3, and 90 ± 4% (average of three independent experiments) in Hep G2 cells pretreated with NAC (30 mM), GSH (30 mM), and V-E (1 mM), respectively. However, NAC, GSH, and V-E, by themselves, had no effect on ERK2 activity. Pretreatment with these free radical scavengers also inhibited tBHQ activation of ERK2 in Hep G2 cells to the same extent as seen with BHA (Fig. 5B). In contrast, pretreatment of Hep G2 cells with NAC and GSH did not substantially change the intensity of JNK1 activation by BHA, although a slight inhibitory effect (about 30%) with vitamin E was observed (Fig. 5C). A similar result was also obtained in HeLa cells with BHA (data not shown). Taken together, these data suggest that ERK2 and JNK1 signaling pathways are differentially regulated. While the generation of reactive intermediates is responsible for the stimulation of ERK2 activity by BHA and tBHQ, JNK1 activation by these two compounds may require a distinct mechanism. This will be discussed later.

Pretreatment with Catalase or Aminotriazole Dramatically Changes the Intensity of ERK2 Activation by H$_2$O$_2$, but Only Moderately Affects ERK2 Activation by BHA and tBHQ—Previous studies have suggested that metabolic formation ROS may be responsible for many biological activities of BHA and tBHQ (22–24). The inhibitory effects of NAC, GSH, and vitamin E, as demonstrated above, also imply the involvement of oxidative stress. To assess the potential role of ROS in regulating BHA- or tBHQ-stimulated ERK2 signaling pathway, we examined the effects of catalase, aminotriazole, and the above free radical scavengers on ERK2 activation by H$_2$O$_2$. Catalase is a highly reactive enzyme and breaks down H$_2$O$_2$ to oxygen and water. Therefore, this enzyme has been widely used to block the biological activities of H$_2$O$_2$ (50). As shown in Fig. 6A, pretreatment of Hep G2 cells with catalase (3000 units/ml) overnight abrogated ERK2 activation by H$_2$O$_2$ (500 μM, 15 min). Consistent with this result, pretreatment with 30 mM aminotriazole, an inhibitor of endogenous catalase, substantially enhanced the ERK2 activation by H$_2$O$_2$. NAC, GSH, and vitamin E also inhibited H$_2$O$_2$ activation of ERK2, although less efficiently than catalase. Interestingly, ascorbic acid (vitamin C), another potent antioxidant, had only a slight inhibitory effect on ERK2 activation by H$_2$O$_2$.

The above results demonstrate that changing the intracellular H$_2$O$_2$ levels by pretreatment with catalase or aminotriazole substantially affected H$_2$O$_2$-triggered ERK2 signaling pathway. If H$_2$O$_2$ serves as a signaling molecule to mediate ERK2 activation by BHA and tBHQ, then pretreatment with catalase or aminotriazole would also show similar effects on BHA- or tBHQ-stimulated ERK2 activity as seen with H$_2$O$_2$. To test this, we pretreated Hep G2 cells either with 3000 units/ml catalase overnight or 30 mM aminotriazole for 60 min, and then stimulated them with BHA (250 μM, 15 min) or tBHQ (500 μM, 30 min). As shown in Fig. 6, B and C, pretreatment with catalase inhibited BHA- or tBHQ-stimulated ERK2 activity, whereas aminotriazole augmented ERK2 activation by the two compounds, implicating the involvement H$_2$O$_2$. However, the inhibitory effect of catalase is much weaker than that seen on ERK2 activation by H$_2$O$_2$. For example, pretreatment with...
3000 units/ml catalase overnight completely abolished ERK2 activation by H$_2$O$_2$, whereas under similar conditions, catalase only moderately reduced ERK2 activation by BHA and tBHQ (about 35 or 10% inhibition was seen for BHA or tBHQ, respectively). Aminotriazole also exerts a weaker effect on ERK2 activation by BHA and tBHQ. Thus, we suspect that H$_2$O$_2$ may not be a signaling mediator, although the changes of H$_2$O$_2$ levels affect ERK2 activation by BHA and tBHQ.

Treatment with BHA or tBHQ Does Not Induce Production of ROS but Rather Causes a Decrease in the Amount of Intracellular ROS—
To determine further the role of H$_2$O$_2$ in BHA- or tBHQ-triggered ERK2 signaling pathway, we measured H$_2$O$_2$-generating activity of BHA and tBHQ using a H$_2$O$_2$-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCF-DA). This chemical is freely permeable to cells. Once inside the cells, it is hydrolyzed to 2',7'-dichlorofluorescein (DCF) and trapped intracellularly. In the presence of peroxides, especially H$_2$O$_2$, DCF is oxidized to fluorescent 2',7'-dichlorofluorescein, which can then be readily detected using fluorescence-activated cell sorting. To validate the assay, an experiment with H$_2$O$_2$ was performed. As shown in Fig. 7A, treatment of Hep G2 cells with 1 mM H$_2$O$_2$ caused an increase in fluorescence intensity as compared with control. Pretreatment with 30 mM aminotriazole for 60 min substantially enhanced the H$_2$O$_2$-activated fluorescence intensity of DCF, although aminotriazole alone slightly increased fluorescence intensity. This is consistent with our earlier observation that pretreatment with aminotriazole potentiated H$_2$O$_2$-stimulated ERK2 activity (Fig. 6A).

We next monitored the intracellular H$_2$O$_2$ level over different periods after exposure of Hep G2 cells to 250 mM BHA or 500 mM tBHQ. To prevent the degradation of H$_2$O$_2$ generated from BHA or tBHQ, cells were pretreated with aminotriazole. As illustrated in Fig. 7B, BHA caused a rapid decrease in intracellular H$_2$O$_2$. The lowest H$_2$O$_2$ levels (about 20% of the control level) were achieved between 5 and 10 min. Afterward, the decreased H$_2$O$_2$ gradually recovered to the control levels. Terefore, both BHA and tBHQ did not show H$_2$O$_2$-generating activities, but instead diminished intracellular H$_2$O$_2$ levels.
Similar results were also obtained with HeLa cells. However, in this cell line, the decrease of H$_2$O$_2$ levels by tBHQ was not as substantial as seen in Hep G2 cells (data not shown). Interestingly, such a cell type-dependent effect of tBHQ is also observed in ERK2 activation by this compound.

**Pretreatment with Ascorbic Acid Abrogates ERK2 Activation by BHA and tBHQ**—The above data clearly indicate that H$_2$O$_2$ is not a signaling mediator for BHA and tBHQ in activation of the ERK2 pathway. We next examined the involvement of other types of radicals. Previous studies have shown that the phenoxyl radical is an unavoidable intermediate in the oxidative metabolism of hindered phenols, and ascorbic acid, although versatile as a scavenger, is the primary reductant of these phenoxyl radicals (51, 52). Thus, we tested the effect of ascorbic acid on ERK2 activation by BHA and tBHQ. Pretreatment with 200 $\mu$M ascorbic acid for 60 min abolished the BHA- and tBHQ-stimulated ERK2 activity (Fig. 8). This inhibitory effect was stronger than that by NAC (30 mM, 60 min). As described earlier, ascorbic acid only weakly inhibited H$_2$O$_2$-stimulated ERK2 activity (Fig. 6A). Thus, this differential inhibitory effect of ascorbic acid further rules out the direct role of H$_2$O$_2$ in BHA and tBHQ activation of ERK2 and implies that phenoxyl radicals generated from the parent molecules may play such a role.

**Sodium Azide, a Peroxidase Inhibitor, Impairs ERK2 Activation by BHA and tBHQ**—The highly inhibitory effect of ascorbic acid prompted us to study further the role of phenoxyl radicals in ERK2 activation by BHA and tBHQ. It is known that one-electron oxidation of phenolic compounds leads to the generation of phenoxyl radicals, and this process has been shown to be catalyzed mainly by peroxidases such as myeloperoxidase and prostaglandin H synthase (53–55). Thus, we examined the potential involvement of peroxidases using a potent peroxidase inhibitor, sodium azide. Pretreatment of Hep G2 cells with 50 $\mu$M sodium azide for 60 min completely attenuated ERK2 activation by tBHQ and, to a lesser extend, by BHA (Fig. 9). This suggests that the reactive intermediates derived from BHA and tBHQ by a peroxidase-dependent metabolism contributed to ERK2 activation by these two phenolic compounds. In addition, the high sensitivity to sodium azide of ERK2 activation by tBHQ may implicate a highly enzyme-dependent mechanism in the formation of radicals from this compound.

**PD98059, a MEK Inhibitor, Potentiates Toxicity of BHA and tBHQ**—To address the potential physiological roles of MAPK activation in the response of cells to BHA and tBHQ, we examined the effects of inhibition of ERK activity on toxicity of BHA and tBHQ in HeLa and Hep G2 cells. Overall, BHA and tBHQ induced a dose-dependent decrease in cell survival in both cell lines (Fig. 10). However, these two compounds at concentrations of 50 and 100 $\mu$M showed a stimulatory effect on cell growth in Hep G2 cells (Fig. 10B). This is in agreement with the observation that BHA and tBHQ at these low concentrations also stimulated ERK2 activity in this cell line (Figs. 1A and 3A). Pretreatment of cells with 25 $\mu$M PD98059, which alone, had no effect on cell survival but greatly potentiated the toxicity of BHA and tBHQ and also abolished the growth-promoting effects of BHA and tBHQ as seen in Hep G2 cells at the low concentrations. In addition, pretreatment with the MEK inhibitor PD98059 did not affect tBHQ-induced cell death in HeLa cells (Fig. 10A), in which tBHQ did not stimulate ERK2 activity (Fig. 3B). Thus, activation of ERK2 by BHA and tBHQ may mediate a process that leads to cell growth or enhances cell survival.

**DISCUSSION**

Since BHA was introduced as a food preservative in the 1960s, it has attracted a lot of attention and debate because of its potential beneficial as well as adverse effects on the health of humans. Although extensive studies have been conducted to define the biological activities of BHA in many animal model
systems and in humans, the mechanisms of action of BHA are not fully understood. In this study, we demonstrated that BHA activates the important signaling kinases, ERK2 and JNK1. The activation of ERK2 was rapid and transient, similar to that reported previously for many growth factors (56, 57). In contrast, JNK1 activation was relatively delayed and sustained, following a pattern similar to that shown by many stress stimuli, such as UVC (37), protein synthesis inhibitors (39), and arsenite (58). Activation of multiple members of MAPK family provides new insights into the action of this ubiquitous food additive.

Although tBHQ, a metabolite of BHA, was also found to activate MAPKs, several lines of evidence indicate that BHA and tBHQ differentially regulate these MAPK cascades. First, BHA strongly stimulates rapid and transient activation of ERK2, whereas tBHQ activation of ERK2 is moderate, delayed, and sustained. Second, BHA activates ERK2 in both HeLa and Hep G2 cells, whereas tBHQ activates ERK2 only in Hep G2 cells, showing a cell line-dependent effect. Furthermore, BHA strongly activates JNK1 in HeLa and Hep G2 cells, whereas tBHQ is a weak inducer of JNK1 activity in both cell lines. Thus, the activation of ERK2 and JNK1 by BHA apparently does not require the conversion to its hydroquinone or quinone metabolites in the cell lines studied.

Activation of MAPKs by BHA or tBHQ raises questions as to the primary signals that trigger these kinase cascades. Our results indicate that formation of oxidative metabolites may play an important role in the activation of ERK2 by BHA and tBHQ, whereas JNK1 activation may require a distinct mechanism. Previous studies have suggested that BHA may undergo mixed-function oxidase-dependent oxidation, resulting in the formation of tBHQ, which has been demonstrated to generate further H$_2$O$_2$, in some in vitro models either by initiating redox cycling or by stimulating NADPH oxidase activity of monoxygenase (9, 22). Therefore, H$_2$O$_2$ has been implicated in the action of BHA and tBHQ. However, we did not observe any production of H$_2$O$_2$ after exposure of cells to BHA and tBHQ using an H$_2$O$_2$-sensitive fluorescent probe. Instead, treatment with BHA or tBHQ substantially reduced the amount of intracellular H$_2$O$_2$. Furthermore, the kinetics and the magnitude of reduction of H$_2$O$_2$ levels are well correlated with the kinetics and the intensity of ERK2 activation by these agents (Fig. 2A, Fig. 3A, and Fig. 7B). Thus, we hypothesized that the oxidative intermediates derived from BHA or tBHQ are responsible for the ERK2 activation, and the formation of such oxidative metabolites can be enhanced by H$_2$O$_2$. This notion is also supported by some other observations as follows: (i) alteration of intracellular H$_2$O$_2$ levels with catalase or aminotriazole affects ERK2 activation by BHA and tBHQ, and (ii) pretreatment with H$_2$O$_2$ at concentrations that do not activate ERK2 potentiates the ERK2 activation by BHA and tBHQ (data not shown). It has been shown that many phenolic compounds, including BHA and tBHQ, are the substrates of peroxidases, such as myeloperoxidases and prostaglandin H synthase, which are known to be present in many tissues (55, 54, 59). Peroxidatic oxidation of BHA or tBHQ leads to the formation of phenoxyl radicals, which can oxidatively modify many biomolecules. In the presence of potent antioxidants such as NAC, GSH, V-E, and especially ascorbic acid, phenoxyl radicals are readily reduced to the parent molecules (51, 52). Thus, peroxidatic-dependent activation of BHA or tBHQ could be a potential mechanism underlying MAPK activation by these compounds. Indeed, pretreatment with the above radical scavengers or a peroxidase inhibitor, such as sodium azide, substantially inhibits ERK2 activation by BHA and tBHQ. Since peroxidatic oxidation of phenolic compounds is also dependent on the availability of H$_2$O$_2$ and other types of peroxides, such as lipid peroxy radicals, modulation of intracellular H$_2$O$_2$ levels would obviously affect the production of phenoxyl radicals and subsequently affect ERK2 activation by BHA and tBHQ, as observed in the present study.

A well-established signaling pathway leading to ERK activation in response to mitogens is the Ras-dependent Raf-MEK-ERK module. Recently, many environmental stress stimuli, such as UVC (60), arsenite (58), and H$_2$O$_2$ (61) were also found to activate ERK through this module. Using a specific inhibitor for MEK, we demonstrated that ERK2 activation by BHA and tBHQ is MEK-dependent. We also observed that BHA and tBHQ induce the autophosphorylation of Raf, indicative of activation of this kinase (data not shown). Thus, it is likely that BHA and tBHQ may use a similar module to stimulate ERK2 activity. How the Raf-MEK-ERK kinase cascade is initiated by BHA or tBHQ is not clear. The oxidative intermediates of BHA and tBHQ, such as phenoxyl radicals, are known to have considerable reactivity with protein thiols, resulting in conformational and functional alteration. The inhibitory effect of V-E suggests that preferential oxidation of membrane-associated proteins might be essential for BHA and tBHQ to initiate ERK2 signaling pathway. Although the oxidative stress may serve as a primary signal for the activation of ERK2, the mechanisms underlying JNK1 activation by BHA are entirely unknown. JNK1 does not seem to be the direct target of BHA because incubation of BHA and JNK1 precipitated with anti-JNK1 antibody does not stimulate kinase activity (data not shown). It has been shown that JNK activation is regulated by a Rac- or Cdc42-dependent kinase cascade, MEKK1/MKK4 (or SEK1, JNKK/JNK), which is parallel to the kinase pathway that regulates ERK2 activation (30). Whether BHA activates JNK1 via this known pathway or through an alternative signaling cascade remains to be determined. It is also interesting to note that BHA stimulates the activities of many lipases, such as phospholipase A2 (62), resulting in the release of arachidonic acid which has been shown recently to be an activator of JNK1 in kidney epithelial cells (63). In addition, BHA has been demonstrated to induce Ca$^{2+}$ influx (64, 65), which is known to initiate many signal transduction events. Thus, future studies focusing on these biochemical steps would extend our understanding of the regulation of JNK signaling cascade simulated by BHA.

What are the physiological consequences of ERK2 and JNK1 activation in response to BHA and tBHQ? ERKs have been commonly associated with cell growth, proliferation, or transformation. For example, activation of ERK2 MAPK is required for mitogenesis, and constitutive elevation of this kinase activity is sufficient to induce cell transformation (66, 67). Consistently, several components of the ERK2 pathway, including Ras, Raf, and MEK, are also shown to have oncogenic potential (68). Our results suggest that ERK2 activation by BHA and tBHQ may lead to the protection against the toxicity of these agents. As shown in Fig. 10, both BHA and tBHQ exerted toxic effects in the cell lines studied. However, in Hep G2 cells, these agents at the lower concentrations, which were sufficient to activate ERK2 and/or JNK1, did not show any toxicity but instead stimulated cell growth. Preferential blockade of ERK2 activation by the MEK inhibitor PD98059 greatly potentiated the toxicity of BHA and tBHQ. Furthermore, since tBHQ did not activate ERK2 in HeLa cells, its toxicity was not affected by the MEK inhibitor. Thus, activation of ERK2 by BHA and tBHQ may actually enhance cell growth or survival. It is tempting to speculate that this growth-stimulating effect of ERK2 activation may also underlie the carcinogenic or tumor-promoting activities of BHA and tBHQ in some animal models of cancer (15, 16). Unlike ERKs, JNKs may mediate more diverse cellular processes, depending on the stimuli. Activation of JNKs has been implicated in inducing apoptosis in response to
growth factor withdrawal and other environmental stimuli (37, 69). Activation of JNKs has also been shown to stimulate cell proliferation and transformation (70). Whether JNK1 activation by BHA is involved in controlling cell survival or death is currently unknown.

Activated ERK2 is known to phosphorylate and activate a ternary complex factor p62TCP/Etk-1, which in turn can interact with the serum response factor to stimulate transcriptional activation of c-fos (71). Activated JNK1 can also phosphorylate and activate many transcription factors, such as c-Jun, ATF2, and TCF/Etk-1, leading to the induction of both c-jun and c-fos (44). Since the products of c-jun and c-fos are key components of transcription factor AP-1, activation of ERK2 and JNK1 by BHA may up-regulate many AP-1-dependent genes. Previous studies have shown that the expression of many phase II detoxifying enzyme genes may be regulated by an AP-1-like element (45). Thus, ERK2 and JNK1 activation may contribute to the anti-carcinogenic activity of BHA by inducing phase II detoxifying enzymes, such as NAD(P)H:quinone reductase, glutathione S-transferase, and UDP-glucuronosyltransferases. The exact biological consequences of ERK2 and JNK1 activation by BHA and tBHQ may be dependent on specific cellular context.

In conclusion, the present study demonstrates that the antioxidant BHA is capable of activating multiple MAPK, independent of its hydroquinone metabolite tBHQ. Oxidative stress due to generation of reactive intermediates, possibly phenoxyl radicals but not H2O2, may be responsible for the activation of ERK2, whereas the JNK1 activation by BHA requires a distinct mechanism. A future challenge will be to define the cellular targets of BHA that trigger signal transduction pathways leading to ERK2 and JNK1 activation and to determine how activation of multiple MAPK mediates diverse biological effects of BHA, such as anti-carcinogenic versus tumor-promoting effects. The understanding of such issues will provide important information on the mechanism of action of phenolic antioxidants.

Acknowledgments—We thank Drs. Anning Lin and Michael Karin (University of California, San Diego, CA) for providing the GST-c-Jun cDNA plasmid; Jie-Jin Jiao for excellent technical assistance; Dr. Judy Bolton for the insightful discussion; and the members of the Kong laboratory for their critical reading of this manuscript.

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