Mitogen-activated Protein Kinase (MAPK) Activation by Butylated Hydroxytoluene Hydroperoxide: Implications for Cellular Survival and Tumor Promotion1

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ABSTRACT

The mitogen-activated protein kinase (MAPK) cascade plays an important role in carcinogenic development. Herein, we show that the skin tumor promoter butylated hydroxytoluene hydroperoxide (BHTOOH) stimulates a rapid and potent (14- to 20-fold) activation of extracellular signal-regulated kinase (ERK) in vivo and in cultured mouse keratinocytes. BHTOOH also moderately (5-fold) activated c-Jun-N-terminal kinase and 38-kDa MAPK-related protein in these same cells. N-acetyl-cysteine and o-phenanthroline abolished ERK activation by BHTOOH, consistent with a requirement for metal-dependent formation of reactive intermediates. Indeed, 4-CD3-BHTOOH, an analogue that generates less of the metabolite BHT-quinone methide (2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone) and fewer tumors in vivo, accordingly exhibited diminished potency for activating ERK. ERK activation by BHTOOH was inhibited by suramin, and by expression of dominant-negative Ras-N-17 in PC12 cells, suggesting overlap between the pathways for BHTOOH and growth factor signaling. Induction of MAPK-dependent genes c-fos and MAPK phosphatase-1 by BHTOOH was also blocked by Ras-N-17 expression. Moreover, expression of Ras-N-17 or kinase-defective MAPK kinase (MEK) diminished cell survival following BHTOOH exposure. Similarly, pretreatment with suramin or the MEK inhibitor PD98059 also potentiated the toxicity of BHTOOH. On the other hand, expression of constitutively active MEK enhanced cell survival. Thus, we demonstrate that the MAPK cascade is critical to the cellular response to BHTOOH. This study suggests a functional role for MAPK activation in tumor promotion stimulated by oxidants and other agents.

INTRODUCTION

BHTOOH (2,6-di-tert-butyl-4-hydroperoxyl-4-methyl-2,5-cyclohexadienone), an oxidative metabolite of the antioxidant food additive BHT, has demonstrated tumor-promoting activity in mouse skin (1). BHTOOH has considerable propensity for generating free radical and electrophilic reactive intermediates (2, 3). Oxidant species such as these are thought to contribute significantly to carcinogenic development and to tumor promotion in particular (4—6). BHTOOH has served as a model tumor promoter to investigate the role of oxidative intermediates in the tumor promotion process. In this regard, BHT-quinone methide (2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone), a reactive electrophile, has been shown to mediate tumor promotion and gene activation by BHTOOH, as well as toxicity by BHTOOH and BHT (3, 7—10). BHT-quinone methide has preferential reactivity with sulphydryl moieties, and cysteine residues in membrane proteins are thought to be particularly subject to covalent modification by this metabolite (7, 11). However, the precise molecular targets of BHT-quinone methide and the signal transduction pathways that control the cellular response to BHTOOH and other oxidant tumor promoters have not been defined.

Activation of the MAPK cascade is essential to signal transduction following exposure to numerous environmental stimuli (12). Several components of the MAPK pathway have demonstrated oncogenic potential in vitro, including Ras, Raf, and MEK; constitutive elevation of MAPK activity has also been demonstrated in certain human tumors (reviewed in Ref. 13; Refs. 14—16). Thus, alterations in the MAPK cascade are clearly implicated in the carcinogenic process, although the function of the MAPK pathway in the tumor promotion stage of carcinogenesis has not been elucidated. To investigate the hypothesis that the MAPK cascade may participate in oxidant tumor promotion, we examined the activation of MAPK by BHTOOH. We demonstrate that treatment of mouse skin in vivo with BHTOOH results in a prominent and transient stimulation of the MAPK family member ERK. Using studies in vitro to more fully explore the regulation of this response, we show convergence in the pathways for activation of ERK by serum and BHTOOH. Moreover, stimulation of ERK by BHTOOH in cultured keratinocytes relies on the formation of BHT-quinone methide, the same reactive intermediate that mediates in vivo tumor promotion by this compound. We further demonstrate that alterations in the ERK pathway, such as may arise in tumor initiation, greatly influence cellular survival following exposure to the tumor promoter BHTOOH. Taken together, our findings indicate that components of the ERK signaling cascade may provide important molecular targets for tumor promoters. This study suggests that mutations arising in the ERK pathway may provide the basis for the differential cellular response to BHTOOH and other oxidants that underlie the tumor promotion process.

MATERIALS AND METHODS

**Chemicals, Cell Culture, and Treatment.** Synthesis and chemical characterization of BHTOOH and 4-CD3-BHTOOH followed the method of Kharasch and Joshi (17) as detailed in Guyton et al. (3). Hydroperoxides were confirmed to be >95% pure from other BHT derivatives by the high-performance liquid chromatography method of Wand and Thompson (18). Cell lines were maintained in a 37°C humidified environment containing 10% CO2 in air. Murine papilloma PE cells (19) were cultured in Eagle minimal essential medium without CaCl2 (BioWhittaker, Walkersville, MD) supplemented with Chexel-treated FCS (8%) and 0.05 mM CaCl2. NIH 3T3 and PC12 cells were cultured in DMEM with gentamicin (50 ng/ml), supplemented with either 10% calf serum (NIH 3T3) or 10% fetal bovine serum and 5% horse serum (PC12). Serum starvation was achieved by incubation in 0.5% serum for at least 16 h before direct addition of BHTOOH into this culture medium. Suramin (0.3 mM), N-acetyl-cysteine (20 mM), or o-phenanthroline (0.1 mM) was added to the culture medium 45 min before direct addition of BHTOOH.

**Immunoprecipitation and Kinase Activity Assays.** Treated cell cultures (60—80% confluent) were washed twice with ice-cold PBS and lysed in kinase buffer (20 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin, 2 μM pepstatin A, 1 mM orthovanadate).
proteins were resolved by SDS-PAGE, and the gels were dried and subjected to autoradiography. The incorporation of 32P was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**BHTOOH Treatment in Vivo.** Female CD-1 mice 4–6 weeks of age (Harlan) in the resting phase of hair growth cycle were treated with 20 μmol BHTOOH. Epidermal lysates were isolated in kinase buffer as described (3). Following normalization of protein content, lysates were incubated for 30 min at 4°C in a suspension of Protein G-Sepharose beads, Protein A-Sepharose beads, and preimmune serum to minimize nonspecific immunoreactive protein content. After centrifugation at 14,000 × g for 10 min, ERK2 was immunoprecipitated from the lysates, and kinase activity was assessed as described above.

**Western Blot Analysis.** Protein extracts were prepared and subjected to Western blot analysis as described previously (21) using a monoclonal antibody specific for ERK2 (Transduction Laboratories, Lexington, KY). Immune complexes were visualized using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) according to the manufacturer’s specificiations.

**Northern Blot Analysis.** Total RNA was isolated from treated cells with Stat60 (Tel Test B, Friendswood, TX), size-separated in agarose/formaldehyde gels, and transferred onto GeneScreen Plus nylon membranes (DuPont NEN, Boston, MA). cDNA probes for c-fos and M KK-1 were labeled with [α-32P]dCTP using a random-primer labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization and washes were performed according to the method of Church and Gilbert (22), and the hybridization signal quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Hybridization to a 24-base oligonucleotide complementary to 18S RNA (5'-ACGGATCTCTGATGCGTCCGAACC-3') that had been 3'-end labeled with [α-32P]dATP by terminal deoxynucleotidyltransferase (Life Technologies, Gaithersburg, MD) was used to control for variation in loading and transfer among samples.

**Cell Viability Assays.** Cells were plated at a density of 50 × 10⁴ cells/well (PC12) or 5 × 10⁵ cells/well (NIH 3T3) into 96-well microtiter plates. The plates were treated with BHTOOH the next day and stained 24 h after BHTOOH treatment with crystal violet for assessment of cell viability using a microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA).

**RESULTS**

**BHTOOH Activates MAPK in Mouse Skin and in Cultured Keratinocytes.** A prominent component of the MAPK family, ERK is highly activated by growth factors and other stimuli (12). Here we examined the effect of in vivo exposure to BHTOOH on ERK activity. BHTOOH (20 μmol) stimulated a rapid and transient rise in ERK kinase activity in mouse skin (Fig. 1A). Maximal ERK activation of 14-fold was found 15 min after in vivo treatment with BHTOOH, with a return to basal ERK levels within 30 min of BHTOOH exposure. Stimulation of ERK kinase activity was not accompanied by a change in the expression of ERK2 protein (Fig. 1B). Instead, enhanced ERK2 activity was paralleled by a shift in the electrophoretic mobility of ERK protein on Western blots (Fig. 1B), consistent with a change in its phosphorylation state.

To facilitate the investigation of pathways leading to ERK activation by BHTOOH, the effect of BHTOOH on MAPK activation was examined in the mouse keratinocyte cell line PE. The kinetics of ERK activation by BHTOOH in cultured keratinocytes are shown in Fig. 2A. Consistent with the time course of in vivo ERK activation by BHTOOH, maximal ERK activation in PE cells was reached within 10–15 min of BHTOOH treatment, whereupon a rapid decline of ERK activity ensued. We also examined the effect of BHTOOH on other MAPK family members. Fig. 2B shows the dose-response relationships for activation of ERK, JNK/SAPK, and p38 in the mouse keratinocyte cell line PE. By comparison with the marked activation of ERK by BHTOOH (>20-fold activation at 80 μM BHTOOH), maximal activation of JNK/SAPK and p38 was only 3- to 5-fold.

**Role of Reactive Intermediates in Initiating ERK Activation by BHTOOH.** BHTOOH undergoes extensive oxidative metabolism to free radical and other reactive intermediates that mediate the biological effects of this compound (2, 3). We therefore sought to explore the role of oxidative metabolites in ERK activation by BHTOOH. As shown in Fig. 3, pretreatment with the glutathione precursor N-acetylcysteine abolished ERK activation by BHTOOH, suggesting that a sulfhydryl-reactive intermediate mediates ERK activation by BHTOOH. The iron scavenger α-phenanthroline also abrogated BHTOOH-stimulated ERK activity, consistent with an iron-dependent mechanism of reactive intermediate formation from BHTOOH.

BHTOOH can be directly activated by heme iron in vitro to reactive species, including free radical metabolites, as well as BHT-quinone methide (2, 3). Comparative analysis of BHTOOH and 4-CD3-BHTOOH enabled investigation of the hypothesis that BHT-quinone methide formation functions in ERK activation by BHTOOH. Because cleavage of a 4-methyl hydrogen is required for formation of BHT-quinone methide from BHTOOH, replacement of the hydrogens in the 4-methyl group with deuterium atoms slows the cleavage of this bond, accordingly reducing quinone methide formation by >2 fold (3). Here, we demonstrate that the capacity of 4-CD3-BHTOOH for ERK activation is diminished in keeping with its reduced ability for BHT-quinone methide formation (Fig. 4). Taken together, the results presented in Figs. 3 and 4 provide evidence that ERK activation by BHTOOH in cultured keratinocytes is reliant on the iron-dependent formation of BHT-quinone methide, the same reactive intermediate known to mediate in vivo tumor promotion by this compound (3).

**Role of Growth Factor Receptors and Ras in ERK Stimulation by BHTOOH.** The phosphorylation cascade resulting in ERK activation by mitogens is initiated by the binding of growth factors to their receptors (reviewed in Ref. 23). This binding is followed by the sequential activation of Ras and Raf (24). Raf then phosphorylates and activates MEK, which directly phosphorylates and activates ERK (25). The growth factor receptor antagonist suramin is known to block
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for c-fos and MKP-1 gene induction by BHTOOH. These findings suggest that modulating the pathway to ERK activation may significantly impact cellular processes that are regulated by ERK-dependent gene expression.

Impact of Altering ERK Activation on the Cytotoxicity of BHTOOH. To explore the functional role of modulating the pathway to ERK activation on cellular outcome following BHTOOH exposure, we examined the influence of cellular Ras status on the cytotoxicity of BHTOOH. As shown in Fig. 7, treatment with BHTOOH mediated a dose-dependent decline in cell survival in both wild-type PC12 cells and those expressing dominant-negative Ras-N-17. However, expression of Ras-N-17 dramatically potentiated the cytotoxicity of BHTOOH. Enhanced sensitivity to BHTOOH was evidenced by a pronounced shift in the dose-response curve for cell survival following BHTOOH treatment. Furthermore, Ras-N-17 expression engendered a reduction in the LC50 of BHTOOH by nearly 4-fold, from 40 μM in wild-type cells to 12 μM in PC12/Ras-N-17 cells.

Although Ras serves a prominent role in ERK signaling, this molecule may directly function in or crosstalk to other signal transduction pathways that may contribute to the overall cellular effects of BHTOOH (24, 34). In particular, Ras has been demonstrated to participate in the activation of the MAPK family member JNK/SAPK (20, 35, 36). As shown in Fig. 8, however, cellular Ras status was without effect on JNK/SAPK activation by BHTOOH. These results are in sharp contrast with the profound influence of Ras-N-17 expression on ERK activation (Fig. 5), MAPK-dependent gene expression (Fig. 6), and cytotoxicity (Fig. 7) stimulated by BHTOOH.
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A. BHTOOH (μM)  
PC12 wt PC12 wt + suramin PC12 Ras-N-17

Activation (fold)  
0 10 20 30 1 5 6 1 4 7

B. Serum (20%)  
PC12 wt + suramin PC12 Ras-N-17

Activation (fold)  
28 6 5

Fig. 5. Common effects of suramin and Ras-N-17 on ERK activation by BHTOOH and serum. Wild-type PC12 cells, wild-type PC12 cells pre-treated with suramin (0.3 μM for 45 min) or PC12/Ras-N-17 cells were treated with the indicated doses of BHTOOH (A) or 20% serum (B). Cells were harvested 10 min later for determination of ERK activity by immune complex kinase assay.

Further evidenced by a reduction in the LC₅₀ of BHTOOH of nearly 2-fold, from 40 μM in control cells to 20–25 μM in the presence of either drug. These results indicate that modulations in the ERK pathway, which result in an altered cellular response to BHTOOH, need not be of a constitutive nature.

DISCUSSION

In most cases, the molecular targets of nonphorbol tumor promoters have not been elucidated (reviewed in Ref. 38). Because these agents stimulate proliferation, components of signal transduction cascades that control the cellular proliferative response are likely candidate molecules. The MAPK family in general, and the ERK cascade in particular, is known to mediate the cellular effects of many environmental stimuli, including mitogens (12). In this study, we demonstrate a rapid and pronounced activation of ERK following in vivo application of the skin tumor promoter BHTOOH (Fig. 1A). That this activation was accompanied temporally by a shift in the electrophoretic mobility of ERK protein, consistent with its phosphorylation, suggests that stimulation of ERK by BHTOOH is mediated by upstream kinases and signal transducers. Indeed, using in vitro studies to more fully explore the regulation of MAPK by BHTOOH, we show further evidence of the specificity of the ERK pathway in controlling cellular survival following BHTOOH exposure, we used three NIH 3T3 cell lines expressing MEKca, MEKca or MEKkd (14). MEK is the immediate upstream regulator of ERK activation, but it is not thought to contribute to the regulation of other MAPK family members and signal transduction modules. In Fig. 9, we demonstrate that MEKkd expression potentiates BHTOOH cytotoxicity, whereas expression of MEKca protects against the cytotoxicity of BHTOOH. The dose-response curves for the cytotoxicity of BHTOOH in these three cell lines are distinct, and the LC₅₀ values for BHTOOH are accordingly disparate (20 μM, 40 μM, and 55 μM in MEKkd, MEKca, and MEKcan cells, respectively). Taken together, these findings suggest that the ERK pathway may serve a pivotal role in determining the cellular outcome following BHTOOH exposure. Indeed, either attenuating or enhancing constitutive MEK expression significantly influences the cytotoxicity of BHTOOH.

Although particularly useful in addressing the molecular basis of BHTOOH sensitivity, constitutive overexpression of dominant-negative or constitutively active expression vectors necessarily has consequent influences on the steady-state cellular environment. We therefore undertook investigation of short-term modulation of the ERK activation pathway on cellular survival following BHTOOH exposure. To this end, we explored the potential sensitizing effects of suramin and PD98059 (37), a compound that specifically inhibits MEK (the immediate upstream regulator of ERK) but not other MEK isoforms that function in other MAPK family signaling pathways. Both of these compounds mediated a shift of the dose-response relationship for the decline in cell survival following BHTOOH exposure (Fig. 10). The potentiating effects of the these inhibitors of the ERK pathway is further evidenced by a reduction in the LC₅₀ of BHTOOH of nearly 2-fold, from 40 μM in control cells to 20–25 μM in the presence of either drug. These results indicate that modulations in the ERK pathway, which result in an altered cellular response to BHTOOH, need not be of a constitutive nature.
that Ras is a critical component of the kinase cascade leading to ERK activation by BHT-quinone methide. Furthermore, suramin could block ERK activation by BHT-quinone methide in PC12 cells indicating that the signal initiates at the cellular membrane (Fig. 5). Thus, there appears to be considerable overlap between the pathway for ERK activation by BHT-quinone methide and those that are normally used by growth factors. These findings are in keeping with the notion that BHTOOH and other tumor promoters may target signaling molecules in the ERK cascade and thereby usurp the pathways normally functioning in the control of the cellular proliferative response.

Free radical species and other oxidative metabolites have been the focus of numerous studies suggesting that such agents play an important role in the carcinogenic process as a whole and in the tumor promotion stage in particular (reviewed in Ref. 5). We have recently demonstrated that free radical species generated from H₂O₂ mediate ERK activation by this agent (28). Additionally, the intracellular production of free radicals has been proposed to participate in gene activation by growth factors, UV radiation, and other stimuli, and more recently, oxidants have been shown to play a role in the stimulation of the kinase cascades that control gene regulation by these agents (26, 39–41; reviewed in Ref. 42). However, the relevance of these findings to in vivo processes such as tumor promotion has not been specifically proven. Using inhibitor studies and structure-activity analyses (Figs. 3 and 4), we demonstrate that ERK activation by BHTOOH is reliant on oxidative metabolism of this compound and that BHT-quinone methide in particular mediates this response. The correlation between the activation of ERK by BHT-quinone methide, and the known tumor-promoting actions of this metabolite (3), suggests a functional role for the ERK pathway in the tumor promotion process.

BHT-quinone methide mediates the cytotoxicity of BHTOOH in cultured keratinocytes, as well as the toxicity of BHT, the parent compound of BHTOOH, in its target tissues (7, 9–11). Taken together with the known tumor-promoting actions of BHT-quinone methide, these studies have suggested that selective toxicity or growth inhibition by BHT-quinone methide may contribute to tumor promotion by BHTOOH and BHT. In such a paradigm, enhanced sensitivity of noninitiated cells to the deleterious effects of tumor promoters would function together with a direct proliferative stimulus to the initiated cell population. In this manner, toxicity to noninitiated cells may provide additional selective pressure contributing to the clonal expansion of individual initiated cells into papillomas (43). Although some studies have suggested that the initiation process confers enhanced resistance to the toxicity of tumor promoters (44), the molecular basis of these changes are not well understood. Here, we demonstrate that expression of dominant-negative Ras or MEK confers enhanced sensitivity to the cytotoxicity of BHTOOH, whereas expression of constitutively active MEK engenders protection (Figs. 7 and 9). Thus, our findings indicate that the ERK pathway serves a protective role against cell death induced by BHTOOH, and that ERK2 activation may in fact function to attenuate the cytotoxicity of BHTOOH. Constitutive alterations in the MAPK cascade, such as may frequently arise in the carcinogenic process (13), may therefore provide a mechanism for the altered response of the initiated cell to extracellular stimuli, including tumor promoters. Importantly, we also demonstrate that short-term inhibition of the ERK pathway can also significantly impact the cellular response to the tumor promoter BHTOOH (Fig. 10). Taken together, these findings suggest a functional role for MAPK activation in the tumor promotion process and provide insight into the molecular targets and mechanisms of action of oxidants and other tumor promoters.

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