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Mechanistic Basis for Inflammation and Tumor Promotion in Lungs of BHT-Treated Mice: Electrophilic Metabolites Alkylate and Inactivate Antioxidant Enzymes

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Abstract

An established model for mechanistic analysis of lung carcinogenesis involves administration of 3-methylcholanthrene to mice followed by several weekly injections of the tumor promoter 2,6-di-*tert*-butyl-4-methylphenol (BHT). BHT is metabolized to quinone methides (QMs) responsible for promoting tumor formation. QMs are strongly electrophilic and readily form adducts with proteins. The goal of the present study was to identify adducted proteins in the lungs of mice injected with BHT and to assess the potential impact of these modifications on tumorigenesis. Cytosolic proteins from treated mouse lungs were separated by two-dimensional electrophoresis, adducts detected by immunoblotting, and proteins identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Eight adducts were detected in the lungs of most, or all, of 6 experimental groups of BALB mice. Of these adducts, several were structural proteins but others, namely peroxiredoxin 6 (Prx6), Cu,Zn-superoxide dismutase (SOD1), carbonyl reductase, and selenium-binding protein 1, have direct or indirect antioxidant functions. When the 9000 *g* supernatant fraction of mouse lung was treated with BHT-QM (2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone), substantial lipid peroxidation and increases in hydrogen peroxide and superoxide formation were observed. Studies with human Prx6 and bovine SOD1 demonstrated inhibition of enzyme activity concomitant with adduct formation. LC-MS/MS analysis of digests of adducted Prx6 demonstrated adduction of both Cys 91 and Cys 47; the latter residue is essential for peroxidatic activity. Analysis of QM-treated bovine SOD1 by matrix-assisted laser desorption ionization-time of flight MS demonstrated predominance of a mono-adduct at His 78. This study provides evidence that indicates Prx6, SOD1 and possibly other antioxidant enzymes in mouse lung are inhibited by BHT-derived QMs leading to enhanced levels of reactive oxygen species and inflammation, and providing a mechanistic basis for the effects of BHT on lung tumorigenesis.

Introduction

In the promotion stage of carcinogenesis, genetically damaged cells acquire a growth advantage over normal cells and form preneoplastic lesions that are susceptible to further genetic damage (i.e., the progression stage) resulting in neoplasia (1,2). The epigenetic nature of promotion renders this process reversible and a promising target for the development of cancer chemoprevention strategies. Promoting agents function by altering gene expression or inhibiting apoptosis. In the former case, promoters influence signal transduction directly by binding to specific cellular receptors or indirectly by encouraging the formation of reactive

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oxygen species (ROS)¹ that alter redox states of signaling molecules (1-3). Much of our knowledge of multistage carcinogenesis has been obtained from studies in murine skin and considerably less information is available about tumor promotion in internal organs.

A well-established model for pulmonary carcinogenesis involves treating mice with an initiator followed by several weekly injections of the food additive 2,6-di-*tert*-butyl-4-methylphenol (BHT) (4). The number of lung tumors in treated animals is 10 to 12-fold higher than in animals receiving only the initiator. Administering BHT alone does not produce tumors (4) but mice develop severe pulmonary inflammation (5) and this response is believed to be a major contributor to promotion as discussed by Bauer et al. (6). In their work, the severity of BHT-induced inflammation correlated with promotion susceptibility in several inbred strains of mice. An unusual feature of this model is that the promoting agent undergoes metabolic activation in the target organ to form electrophilic metabolites that mediate its effects (7-10). The *para*-quinone methides BHTQM and BHTOH-QM shown in Figure 1 undergo 1,6-Michael additions to thiol groups of Cys and (less rapidly) to amino groups of Lys and His residues (11,12), but specific protein targets in lungs are unknown. In recent work, proteomics methodologies involving 2-dimensional SDS-PAGE (2-DE) separations of proteins, immunochemical detection of adducts with polyclonal antibodies responsive to both BHT and BHTOH groups, and identifications by LC-MS/MS were utilized to elucidate targets in lung epithelial cells treated directly with these QMs (12,13). A total of 37 immunoreactive proteins were identified in cells, including several that influence cell growth or participate in stress responses. In the present study, the same methodology was applied to identify adducts formed in the lungs of mice injected with BHT. Six different treatment protocols were used. Although adducts of more than 20 proteins were identified, we focused on 8 proteins that were alkylated in cytosols from four or more treatment groups. Several of the identified proteins are antioxidant enzymes and their impaired activity may be the basis for oxidative stress detected in earlier studies when pulmonary epithelial cells were exposed to the QMs (14)

Extensive data demonstrate a causative role for oxidative stress in the promotion phase of carcinogenesis. ROS and H₂O₂ influence cell signaling by oxidizing Cys residues in kinases, phosphatases, and transcription factors (3,15). One of the principal QM targets identified in the present work is cytosolic peroxiredoxin 6 (Prx6), an enzyme that, like catalase and glutathione peroxidase, controls intracellular levels of H₂O₂ (16). In addition, Cu,Zn superoxide dismutase (SOD1) was also adducted in BHT-treated mice. This soluble cytoplasmic protein catalyzes the conversion of superoxide anion radicals to molecular oxygen and hydrogen peroxide. The sites of alkylation and the influence of alkylation on activity of these two enzymes were investigated *in vitro*. Our results suggest that BHT-derived QMs, and perhaps many other electrophilic xenobiotics or their metabolites, play a significant role in epigenetic aspects of carcinogenesis through alkylation and inhibition of protective enzymes.

Materials and Methods

Chemicals

All chemicals were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) unless otherwise noted. Solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA). BHT was recrystallized from hexane and BHTOH was synthesized previously (11). BHT-QM and

¹Abbreviations: 2-DE, two-dimensional isoelectric focusing SDS-PAGE; BHT, 2,6-di-*tert*-butyl-4-methylphenol; BHTOH, 6-*tert*-butyl-2-(1',1'-dimethyl-2'-hydroxy)ethyl-4-methylphenol; CID, collision-induced dissociation; CR, carbonyl reductase; GSTP1, glutathione S-transferase P1; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MeCN, acetonitrile; MS/MS, tandem MS; MSDB, Mass Spectrometry Data Base; Prx6, peroxiredoxin 6; PVDF, poly(vinylidene difluoride); QM, quinone methide; ROS, reactive oxygen species; S9, 9000 g supernatant fraction; SBP1, selenium-binding protein 1; SOD1, Cu,Zn-superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TBHP, *tert*-butyl hydroperoxide.

BHTOH-QM were prepared by oxidizing the corresponding phenol with silver oxide in acetonitrile (MeCN); the concentration was determined by UV analysis as described (9,10).

Animals and Treatments

Male BALB/cByJ mice were obtained from Jackson Laboratory (Bar Harbor, ME) at 4 to 6 weeks of age and acclimated for 1 week prior to treatment. Animals were randomly distributed into six groups of 5 animals each. Each mouse, weighing approximately 25 g, was injected ip with a dose of 150 mg of BHT/kg body weight delivered in 100 μ L of corn oil. All mice received the first injection at the same time and some groups received multiple injections at later time points as summarized in Table 1; mice from each group were euthanized on the day indicated in the table. Lungs were removed and the pooled tissue from 5 mice homogenized in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA as described (7). Some of the supernatants produced by centrifugation at 9,000 g for 20 min (S9 fractions) were centrifuged again at 100,000 g for 60 min to yield cytosolic fractions. Protein concentrations were determined using the Bradford assay (17). Samples were flash frozen in liquid nitrogen and maintained at -80°C until analysis.

Adduct Detection

Immunochemical procedures for detection of protein adducts have been described in detail (13). Briefly, cytosols containing 2 mg of protein were concentrated with a Centricon YM10 ultra filtration cell (Millipore, Billerica, MA) to a volume of approximately 50 μ L, desalted with a BioSpin 6 gel filtration column (BioRad, Hercules, CA) and exchanged into 1 mM sodium phosphate buffer at pH 7.4. The volume was reduced to approximately 20 μ L by lyophilization and divided into two equal samples. Each sample was added to 600 μ L of Amersham Destreak rehydration buffer (GE Healthcare, Piscataway, NJ) and 2 μ g of BHT-QM-adducted soybean trypsin inhibitor prepared as described was added (13). Protein samples were processed in the first dimension by isoelectric focusing (60,000 v-h) on either pH 3–10 or pH 5–8 Ready Strips (17 cm, BioRad), and in the second dimension on 4–15% linear gradient slab gels (18.5 $\times$ 20 cm). Proteins were either stained in the gel with Coomassie Brilliant Blue R250 in 40% methanol/10% acetic acid overnight or were electroblotted to a PVDF membrane (0.45 μ M) in a semi-dry transfer apparatus with constant voltage maintained at 15 V for 60 min. Once transfer was complete, membranes were blocked overnight with a solution of 5% non-fat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.1% Tween 20), probed with the primary antiserum (1:500), washed with TBST, probed with anti-rabbit IgG linked to horseradish peroxidase, and visualized by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA) with exposure times of 1 min onto Hyperfilm ECL (GE Healthcare).

Protein Identification

Adducts detected on western blots were carefully matched to the corresponding spots on Coomassie-stained companion gels utilizing BHT-adducted soybean trypsin inhibitor together with adducted carbonyl reductase (CR) and albumin present in all lung samples to facilitate alignments as described (13). Spots were excised from the gels, treated with dithiothreitol, cysteines alkylated with iodoacetamide, proteins hydrolyzed with modified porcine trypsin (Promega, Madison, WI) in 100 μ M of ammonium bicarbonate, and the resulting peptides prepared for MS analysis as detailed previously (13). Samples were introduced into an Esquire HCT ion trap mass spectrometer (Bruker, Billerica, MA) with an electrospray source, and an 1100 capillary HPLC system (Agilent, Palo Alto, CA) with a 1.0 $\times$ 150 mm Jupiter Proteo 90 $\text{\AA}$ column (Phenomenex, Torrance, CA). The flow rate was 50 μ L/min of 0.1% formic acid in MeCN (solvent A) and 0.1% aqueous formic acid (solvent B). Mobile phase composition was held at 3% solvent A in solvent B for 5 min and then increased to 40% A in a linear gradient

over 40 min. In some cases a 0.30×150 mm PepMap C18–100 Å column (LC Packings, Sunnyvale, CA) was utilized at a flow rate of 5.0 $\mu\text{L}/\text{min}$ with the same solvent program. The mass spectrometer was operated with skimmer and capillary exit voltages of 40 and 140 V, respectively, under auto MS/MS conditions utilizing parameters established by the Bruker software. The data were exported to Mascot Generic Format files and searched against the comprehensive non-identical Mass Spectrometry Database (MSDB) obtained from <ftp.ncbi.nih.gov/repository/MSDB> via Mascot (www.matrixscience.com). Search parameters were: delta mass 0.6 Da, one missed cleavage, and optional modifications for oxidized Met, carbamidomethyl-Cys, and BHT-QM-adducted Cys, Lys, or His. In some cases, proteolytic digests also were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS on a Bruker Omnisflex instrument. Those samples were prepared by adding peptides in 1:1 MeCN-water with 0.1% trifluoroacetic acid to an equal volume of a saturated solution of -cyano-4-hydroxycinnamic acid in 1:1 MeCN-water containing 0.05% trifluoroacetic acid, spotting 1 μL on the MALDI plate, and allowing crystals to form. Internal mass calibrations were conducted with peptide standards (Sigma) and the data analyzed with Bruker Biotoools software.

Measurements of Lipid Peroxidation and ROS

- i. Thiobarbituric acid-reactive substances (TBARS) were measured in the S9 fractions of lungs from untreated mice as described (14). Typical reactions were conducted with 500 μL of PBS at pH 7.4 containing cytosol (13 mg protein/mL) and 50, 100, or 200 μM BHT-QM delivered in MeCN solution to produced a final solvent concentration of 2% v/v. Incubations were conducted at 37 °C for 30 min and then treated with 500 μL of a solution containing 30% trichloroacetic acid and 0.73% thiobarbituric acid. These solutions were incubated at 100 °C in a boiling water bath for 15 min, cooled in the dark, centrifuged at 2000 g for 10 min, and absorbance at 535 nm determined. Some samples were incubated at 37 °C for 10 min with 1.0 mM TBHP or 2.0 mM diethyl maleate prior to addition of BHT-QM. Incubation with BHT rather than BHT-QM produced no increase in TBARS.
- ii. Superoxide was measured in S9 fractions of mouse lung diluted to 1.0 mg/mL protein in 50 mM potassium phosphate buffer and 10 mM KCl at pH 7.4. Samples were incubated at 25 °C in a thermostated cuvette in the presence of 50 or 100 μM BHT-QM and controls contained MeCN only in a total volume of 0.85 mL. After 30 min, partially acetylated cytochrome C was added to 27 nmol and a baseline absorbance established at 550 nm over a 1-min time period. NADPH was added to 0.38 μmol and absorbance measured over the next 200 s, then bovine SOD1 was added to 0.5 nmol and the absorbance was measured for an additional 200 s. Superoxide formation was calculated as the SOD-dependent absorbance change using an extinction coefficient of $21 \text{ cm}^{-1} \text{ mM}^{-1}$ as described (14,18).
- iii. Hydrogen peroxide was measured using a colorimetric assay kit (product CS0270, Sigma) based on ferrous ion oxidation in the presence of xylenol orange with absorbances determined at 550 nm. A standard curve was generated for H_2O_2 over the range 0.10–3.50 $\mu\text{g}/\text{mL}$. Incubations were conducted in a total volume 100 μL containing lung S9 fractions (1.0 mg protein/mL) in potassium phosphate buffer (pH 7.4), and 0, 50, 100, or 200 μM BHT-QM at 37 °C for 30 min. Samples were then diluted 64-fold with phosphate buffer (pH 6.0) and 50 μL of this solution was added to 100 μL of the colorimetric reagent in micoplate wells. Reaction was allowed to occur for 30 min at room temperature and the absorbances were measured.

Enzyme Activities

- i. *Prx6*. Activity of human recombinant Prx6 (Lab Frontier, Seoul, Korea) was measured as essentially as described (19) in an assay adapted to 250 μ L volumes and 10 μ g of protein in a microplate format. Activity was measured by the consumption of NADPH in the presence of GSH and GSH reductase. A typical reaction contained 50 mM potassium phosphate buffer (pH 8.0), 2 mM sodium azide, 0.1 mM EDTA, 0.3 mM NADPH, 0.36 mM GSH, and 0.23 units/mL GSH reductase. To determine the effects of QM on activity, 1 mg/ml of protein was incubated with various concentrations of BHT-QM (in the range of 20–100 μ M) or BHTOH-QM (10–50 μ M) delivered in MeCN (2% v/v) at 37 °C for 30 min in 1.0 mL of 50 mM potassium phosphate buffer, pH 7.4. Time course experiments were conducted with 75 μ M BHT-QM or 35 μ M BHTOH-QM at various times over the range 5–30 min. Each treated sample was diluted 100-fold for activity assays. Control experiments are described in the Results section.
- ii. *SOD1*. Solutions containing 2.5 μ g of bovine erythrocyte SOD1 in 250 μ L of 50 mM potassium phosphate buffer (pH 7.4) were incubated with 50 or 200 μ M BHT-QM (added in 5 μ L of MeCN) or with the vehicle only (untreated control) at 37 °C for 30 min. Samples were then diluted to 1.0 mL with water and SOD1 activity measured spectrophotometrically at 37 °C utilizing a BIOOXYTECH® SOD-525 kit from OxisResearch (Portland, OR) according to the manufacturer's instructions. This assay determines activity by measuring the SOD1-mediated enhancement in the rate of autooxidation of a tetracyclic catechol to form a chromophore absorbing at 525 nm, and is susceptible to interference from reducing agents such as BHT (20). BHT is a contaminant in synthetic BHT-QM solutions, and so a separate set of samples was analyzed with BHT substituted for BHT-QM.

Preparation and analysis of Prx6 and SOD1 adducts

Human Prx6 or bovine SOD1 (10 μ g) dissolved in 50 μ L of phosphate buffer (pH 7.4) were incubated at 37 °C for 30 min with the vehicle only (MeCN, 5 % v/v) or with BHT-QM to give QM:protein molar ratios of 1, 5, and 10. Following incubation, 10 μ L of each solution was mixed with an equal volume of 2X SDS-PAGE sample preparation buffer and analyzed on a 10% gel (13). A duplicate gel was analyzed by western blotting with the antiserum described above. Bands corresponding to Prx6 treated with an equimolar amount of BHT-QM were excised from the Coomassie-stained gel, hydrolyzed with trypsin and analyzed by LC-MS/MS as described above. In studies of SOD1 adduction, 0.1 mg of the protein in 100 μ L of 10 mM ammonium bicarbonate was treated with a 10-fold molar excess of BHT-QM, or with the vehicle (MeCN) only, and incubated at 37 °C for 30 min. The reaction mixture was lyophilized to dryness and the residue redissolved in 1 mL of 0.1% trifluoroacetic acid and 1 μ L analyzed by MALDI-TOF MS using sinapinic acid as the matrix and apomyoglobin for external mass calibration. Other samples containing 3 μ g of adducted SOD1 were hydrolyzed as described above for the other proteins, except that Asp-N (49 ng) in 1 mL of 10 mM Tris-HCl buffer (pH 8.0) was added instead of trypsin and solutions were incubated at 37 °C for 20 h (21). Digests were analyzed by both LC-MS/MS and MALDI-TOF MS using the conditions described under "Protein Identification."

To examine zinc release from SOD1 after treatment with BHT-QM, solutions were concentrated to a volume of 1.5 mL by lyophilization and then passed through a Centricon YM-3 filter (Millipore) to remove the protein. The filtrate was lyophilized to dryness, redissolved in 1.5 mL of purified, deionized water and analyzed for zinc content with an atomic absorption spectrometer fitted with a deuterium-arc background correction lamp (Analyst,

Perkin Elmer, Shelton, CT). Samples were aspirated to the flame and the SD was <3 % of the mean (n = 3) in each case.

Results

Identification of Protein Adducts

Groups of BALB mice were treated with BHT using six different protocols (Table 1) ranging from a single injection, sufficient to produce pulmonary inflammation, to four weekly injections required for tumor promotion (4,6). The objectives were to identify potentially important protein adducts in lung cytosols and to determine how soon after injection these adducts appeared and how long they persisted. Lungs were removed and cytosolic proteins analyzed by 2-DE and western immunoblotting with antiserum that recognized BHT and BHTOH groups attached to proteins by thioether or amine linkages (13). A representative blot shown in Figure 2 contains 24 clearly defined spots; these spots correspond to the proteins identified by LC-MS/MS and listed in the figure legend. Multiple forms of the same protein, presumably representing different post-translational modifications, were observed in some cases. Several adducts were detected in only one or two of the six treatment groups, so those data must be considered preliminary until verified by additional work. The eight proteins listed in Table 2, however, were detected in four or more of the six experimental groups and were reliably identified by Mascot searches of the MSDB. The resulting Mowse scores were >300 in each case, far exceeding the minimum acceptable score of 65 (www.matrixscience.com). Among these proteins, five were adducted at detectable levels in lung cytosol just 24 h after a single injection of BHT (Group I). All eight adducts were detected after 3 days (Group II) and after 28 days (Group VI). The failure to detect four adducts at an intermediate time point (i.e. in Group IV or V) is more likely due to experimental variation in multi-step sample preparation and analysis procedures than a failure to be produced in that particular treatment group. Positive results are clearly much more meaningful than negative results in these studies. Several of the proteins detected as adducts—Prx6, SOD1, CR, and selenium-binding protein 1 (SBP1)—are directly or indirectly involved in antioxidant functions and are of particular interest as their protective roles may be compromised by alkylation.

Oxidative Stress and Inhibition of Antioxidant Enzymes *In Vitro*

The ability of BHT-QM to induce oxidative stress was examined by incubating the QM with S9 fractions from lungs of untreated mice. As shown in Table 3, substantial lipid peroxidation occurred: at 50 μ M BHT-QM, TBARS production was increased 4-fold compared with untreated controls. Samples treated with 100 μ M BHT-QM underwent nearly twice as much lipid peroxidation as controls treated with TBHP at a 10-fold higher concentration. Treatment of S9 fractions with diethyl maleate (Table 3 footnote), in order to lower levels of endogenous GSH responsible for trapping a portion of the QM (14), produced an additional 30% increase in lipid peroxidation relative to the untreated control. Levels of both O_2^- and H_2O_2 in lung S9 incubates also were increased by several-fold after treatment with BHT-QM (Table 3). These results demonstrate the ability of the QMs to enhance the formation of ROS and induce oxidative stress, consistent with the inactivation of antioxidant enzymes.

Human Prx6 is an excellent model for the murine protein with approximately 90% sequence homology. Proteins from both species contain two Cys residues, one of which (Cys 47) is conserved in the catalytic site and participates in the reduction of H_2O_2 and lipid hydroperoxides (16,22). Western blotting of human Prx6 treated with increasing amounts of BHT-QM confirmed adduct formation; whereas the intensities of Coomassie-stained bands were unaffected, the intensities of immunoblots increased up to a 5-fold molar ratio of QM:protein (Figure 3). Neither the vehicle alone nor 50 μ M BHT altered Prx6 activity whereas 50 μ M BHT-QM lowered activity by 45% (Table 4). Inhibition was not affected by dialysis

of the treated protein, but was prevented by the addition of N-acetylcysteine to efficiently trap BHT-QM as a thioether conjugate. The extent of Prx6 inhibition by the QM depended both incubation time and electrophile concentration as shown in Figures 4A and 4B. In addition, BHTOH-QM was a more effective inhibitor than BHT-QM consistent with higher reactivity demonstrated previously for the hydroxylated derivative (10).

Bovine SOD1 was utilized as a model of the murine protein because both contain three Cys residues in homologous positions (i.e., Cys 6, 55, and 144 in bovine SOD1 and Cys 6, 57, and 146 in the murine protein) (23). As with Prx6, treatment of SOD1 with increasing amounts of BHT-QM produced more extensively alkylated protein up to a QM:protein ratio of 5:1 (Figure 3) and decreasing activity (Figure 4C). Control incubations with BHT were conducted because this compound is present in solutions of synthetic BHT-QM and is known to interfere with the colorimetric assay utilized to measure SOD activity (20). Our data demonstrate that at BHT concentrations comparable to BHT-QM, a relatively modest effect was observed that does not account for the majority of inhibition seen with the QM.

Alkylation Sites in Prx6 and SOD1

To determine the basis for enzyme inhibition, QM-treated Prx6 was excised from a Coomassie-stained gel, digested with trypsin, and analyzed by LCMS/MS. Two adducts were detected among these peptides (labeled I and II in Figure 5A) through neutral losses of BHT-QM upon collision-induced dissociation (CID) (Figure 5B). Facile cleavage of the QM from these thioether adducts (12, 13) necessitated MS³ analysis in order to confirm peptide sequences. Adduct I consisted of the residues 85–97 corresponding to peptide DINAYNC⁹¹EEPTEK and adduct II matched residues 42–53 corresponding to the sequence DFTPVC⁴⁷TTELGR. The positions of alkylation were assumed to be at the most reactive sites, i.e. Cys 47 and Cys 91. The -amino group of Lys 97 is the only other possible site of adduction, but in addition to a substantial preference for reacting with thiols, QM alkylation of Lys 97 would have blocked tryptic cleavage.

Adduction of bovine SOD1 by BHT-QM was demonstrated by western immunoblotting (Figure 3B). Digestion of protein pre-treated with a 10-fold molar excess of BHT-QM was carried out using endoproteinase Asp-N that produced higher coverages than trypsin as reported previously (21). No peptide adducts were detected, however, during LC-MS/MS analyses of the digests. On the other hand, MALDI-TOF analysis of the treated, intact protein confirmed adduct formation as shown in Figure 6A. The average mass of the native apoprotein was shifted from 15,592 Da (theoretical mass 15,591) to 15,811 Da corresponding to a mono adduct. A small amount of a di-adduct also was present at 16,031 Da, and the peak area ratios of non-adducted protein to mono- to di-adducted protein were 1.0 : 0.9 : 0.2. MALDI-TOF spectra of the Asp-N digest of this mixture contained 10 peptides including one (at 1729.8 Da) with a BHT-QM group attached (Figures 6B and 6C). This peptide corresponded to residues 74–87, i.e. peptide DEERH⁷⁸VGD LGNVTA, presumed to be adducted at His 78 as the imidazole side chain is the only site sufficiently nucleophilic to react with the BHT-QM (11, 13). A larger peptide (at 3357.63 Da) containing a free His 78 residue also was detected in lower abundance, demonstrating incomplete adduction as expected from Figure 6A. Peptides containing the three Cys residues (Cys 6, 55, and 144) without a QM group attached also were detected in the digest. The results suggest that in SOD1, imidazole nitrogens rather than Cys thiols are targeted by BHT-QM. The three Cys residues are known to be resistant to alkylation as Cys 55 and Cys 144 form an intramolecular disulfide linkage and Cys 6 is located near the homodimer interface (25).

The results suggest that His 78, one of the four His residues involved in zinc ligation, may be the primary site of alkylation and that inhibition of enzyme activity may be due to the disruption of zinc binding. To test this possibility, SOD1 was treated with a 10-fold excess of BHT-QM

and adduct formation confirmed by MALDI-TOF analysis of intact protein. The solution was then passed through a 3-kD molecular weight cut-off filter producing a protein-free filtrate, and the latter analyzed by atomic absorption spectrometry for the presence of unbound zinc. These solutions contained barely detectable levels of zinc (approximately 0.03 $\mu\text{g/mL}$) and were indistinguishable from negative controls in which SOD1 was treated only with MeCN (the vehicle). In contrast, a positive control conducted by treating SOD1 with 1% trifluoroacetic acid, produced 6.41 $\mu\text{g/mL}$ of free zinc in the filtrate corresponding to 77% of the theoretical total. These results demonstrate that QM adduction of SOD1 is not accompanied by the release of zinc.

Discussion

Our goals were to identify protein adducts generated in lungs of mice treated with BHT and to determine which of these modifications are most likely to contribute to tumor promotion. Eight of the proteins detected on 2-DE immunoblots (Table 2) were present in cytosols from all or most of six treatment groups and three proteins—Prx6, SOD1, and CR—protect cells from ROS and/or reactive products of lipid peroxidation. In addition, a fourth adducted protein (SBP1) may have antioxidant functions. Prx6 is of particular interest as it is highly expressed in lung and has a role in maintaining the appropriate intracellular levels of H_2O_2 (16,26). This enzyme catalyses both peroxidase and phospholipase A_2 reactions at different active sites and is termed a 1-Cys Prx because of a single conserved Cys residue in the peroxidatic site (Cys 47 in the human enzyme) (22,27). The human and murine forms of Prx6 also contain a second Cys residue near the protein surface (i.e., Cys 91 in the human form). Cys 47 is directly involved in the reduction of peroxy bonds of H_2O_2 and lipid-derived hydroperoxides forming, respectively, water and an alcohol. The present results demonstrate that human Prx6 is highly sensitive to inhibition by QMs; approximately 50% of the activity of the human enzyme was destroyed by treatment with 50 μM BHT-QM and 80% was lost after treatment with 50 μM BHTOH-QM (Figure 4B). The levels of H_2O_2 were dramatically increased in S9 fractions from mouse lung exposed to BHT-QM (Table 3), consistent with Prx6 inhibition, although there may be additional QM targets in the S9 fraction that contributed to this effect. Clearly, elevated H_2O_2 may lead to increased lipid peroxidation in lung S9 fractions treated with BHT-QM.

Attempts to identify specific alkylated peptides in digests of Prx6 isolated from lung cytosols were not successful; some of the difficulties normally associated with mass spectrometric detection of protein adducts from cells and tissues have been discussed previously (13). QM alkylation sites were characterized, however, by LC-MS/MS analysis of QM-treated purified human Prx6. Peptides containing a BHT group and also containing either Cys 47 or Cys 91 were identified in tryptic digests. In each case, CID resulted in a facile neutral loss of BHT-QM, not unexpected because of the labile nature of BHT-thioether adducts (12,13). Other examples of Prx6 adduction are known, for example in lung by epoxides formed during the metabolism of naphthalene (28). To our knowledge, however, the present study is the first to demonstrate alkylation of the catalytic Cys 47 residue by an electrophilic metabolite accompanied by enzyme inhibition. This residue is located in the interior of the protein and it has been suggested that protein unfolding occurs to enable substrate access (29). Given that lipid hydroperoxides also are Prx6 substrates, it is possible that BHT-QM gains access to the catalytic Cys 47 because it is also strongly hydrophobic.

Another factor that may contribute to Prx6 inhibition *in vivo* is alkylation of GSTP1. It was reported that the latter is involved in regenerating the reduced (i.e., active) peroxidase during the Prx6 catalytic cycle (29). In earlier work, we demonstrated adduction of GSTP1 by BHT-QM in a transformed cell line derived from mouse lung epithelium, and confirmed that alkylation of Cys residues destroyed conjugation activity (12). The most abundant form of GSH S-transferases in mouse lung, GSTM1 (31), was detected in immunoreactive 2-DE spots

from cytosols of two treatment groups (data not shown), but no GSTP1 adduct was found. However, GSTP1 is expressed at higher levels in tumors than in non-tumor tissue (30) so adducts may have been present at undetectable levels in the normal lungs examined here. Taken together, these data indicate that alkylation and inhibition of pulmonary Prx6 (and possibly GSTP1) by BHT-derived QMs resulted in increased levels of H₂O₂ leading to oxidative damage and, presumably, to alterations in cell signaling that play key roles in tumor development (15,32).

Adducts of SOD1 also were detected in lungs of BHT-treated mice and were evaluated *in vitro* after treatment of purified bovine protein with BHT-QM. Inhibition occurred to a lesser degree than for Prx6. SOD1 activity decreased by about 10% at 50 μ M BHT-QM and about 60% at 200 μ M BHT-QM, compared to control values (Figure 4). The rate of formation of O₂⁻ in BHT-QM-treated lung S9 fractions was higher than in untreated S9, consistent with impaired SOD1 activity. MALDI-TOF analysis of the intact protein treated with a 10-fold excess of BHT-QM demonstrated approximately 50% conversion to a mixture of mono- and di-adducts with the former predominating (Figure 6). Analysis of an Asp-N digest revealed a single adducted peptide consisting of residues 74–87 with a mass increment of 218 Da over its theoretical mass corresponding to the addition of BHT-QM. The most likely site of attachment is the imidazole ring of His 78 (11).

Residues His 61, 69, and 78, and Asp 81 of bovine SOD1 are involved in zinc ligation (23). We initially speculated that alkylation of the imidazole group of His 78 inhibited enzyme activity by disrupting zinc binding. Several attempts to detect release of the metal from QM-treated protein by atomic absorption spectroscopy demonstrated that the metal remained bound to the protein despite alkylation. It may be that alkylation of His 78 is sufficient to affect enzyme activity but not to dislodge zinc from the protein, or alternatively that His 78 is actually a minor alkylation site and another, more important site was not detected. Interestingly, the proteolytic fragment containing His 41, the only metal-free His (33), produced a very intense MALDI-TOF ion at *m/z* 1180 Da demonstrating that this residue was not appreciably alkylated. In summary, the alkylation of SOD1 in lungs of BHT-treated mice is strongly supported by immunochemical and MS detection of the adduct in 4 out of 6 treatment groups and by immunochemical and MS studies of the closely related bovine enzyme treated with BHT-QM. Inhibition of SOD1 by treatment with BHT-QM was clearly demonstrated with the bovine enzyme and further supported by increased rates of O₂⁻ formation in NADPH-fortified lung S9 treated with BHT-QM. In contrast to strong evidence implicating Prx6 alkylation in the actions of BHT, however, the contribution of SOD1 inhibition to oxidative damage and the specific mechanism of inhibition remain to be clarified.

Two additional proteins listed in Table 2, CR and SBP1, may also contribute to the pulmonary effects of BHT. Carbonyl reductases are highly expressed in lungs and activity is substantially higher in tumors than in normal tissue (34,35). These enzymes catalyze the reduction of endogenous and exogenous aldehydes and ketones, including products of lipid peroxidation (34), and a role for CR in antioxidant protection has been demonstrated (36). Although some forms of CR contain critical Cys residues in the substrate or cofactor binding sites, the mouse protein contains only two Cys residues located near the solvent surface (refer to PDB entry 1CYD) (37). If alkylation occurs on these residues, formation of the active homotetrameric form of the enzyme may be hindered causing a loss of activity. Inhibition of CR could also occur if a critical amine-containing residue is alkylated, for example Lys 17 or Lys 153 involved in NADPH binding (37). Regardless of the specific mechanism involved, QM-induced inhibition of CR could exacerbate cellular damage resulting from oxidative stress and lipid peroxidation.

The functions of selenium binding protein are not fully understood. The closely related forms SBP1 and SBP2 are highly expressed in many tissues, contain several Cys residues, and are commonly targeted by electrophiles including metabolites of acetaminophen and naphthalene (28,38). SBP1 expression is lower in lung adenocarcinomas than in normal tissue and downregulation has been correlated with low survival rates for cancer patients (39). Expression of SBP2 decreases in response to cell proliferation induced by clofibrate and other peroxisome proliferators (40,41). The apparent growth-inhibitory characteristics of SBPs indicate an anticarcinogenesis role for these proteins. SBPs are believed to participate in the intracellular transport of selenium and downregulation is expected to disrupt selenium metabolism. In addition, direct antioxidant activity has been postulated for SBP in analogy to other selenocysteine-containing enzymes such as thioredoxin reductase and GSH peroxidase (42). Adduction of SBP1 may interfere with its normal functions, effectively mimicking downregulation and the associated loss of growth inhibitory and other protective functions.

Several protein targets of BHT-derived QMs discussed above have roles in protecting cells from oxidative stress, and their inactivation provides a mechanistic basis for understanding pulmonary inflammation and tumorigenesis in mice treated with BHT. The most compelling data implicating protein adduction in the underlying mechanisms concern Prx6 inhibition. We currently have no evidence that QMs alkylate other peroxidases (i.e., catalase and glutathione peroxidase); however, the important role of peroxiredoxins in metabolizing H₂O₂ has become clear in recent years (27). In addition, the role of H₂O₂ in cell signaling is well established (15,32,43). This oxidant influences the redox states of Cys residues in signaling molecules, including transcription factors, kinases, and phosphatases, with consequences on cell growth, survival, and apoptosis. The finding that Prx6 is rapidly adducted following the first dose of BHT is consistent with the rapid onset of pulmonary inflammation (5). Adducted Prx6 was detected in lung cytosols 24 hours after treatment with BHT and throughout the 4-week treatment period necessary for tumor promotion, indicating that a period of sustained oxidative stress is required to induce cell proliferation and the resulting accumulation of preneoplastic lesions.

The abundant cytoskeletal proteins tropomyosin, annexin A3, and β -actin were also alkylated in most or all of the treatment groups (Table 2); however, none of these proteins are likely to play a role in the effects of BHT on tumorigenesis. Only protein adducts present in relatively high amounts were detected in this work, but less abundant proteins related to signal transduction, including kinases and transcription factors, have Cys residues that may also be alkylated providing a direct route for the modification of cell signaling pathways (44). The data presently in hand, however, support a state of persistent oxidative stress over several weeks as the major cause of cell proliferation. Other than QMs derived from BHT or structurally-similar alkylphenols (8,10) and an electrophilic metabolite of safrole (45), there is a paucity of specific examples of tumor promotion by electrophiles. Nevertheless, it is probable that other electrophiles of exogenous or endogenous origin influence tumor development by epigenetic mechanisms, especially if exposures occur over a prolonged period of time.

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Figure 1.
Structures of BHT, the metabolites discussed in the text, and protein adduct formation.

Figure 2.

Western blots of cytosolic proteins from mouse lung. (A) Cytosol from the lungs of mice in treatment Group III. A total of 24 spots were excised from the companion 2-DE gel and the resulting digests analyzed by LC-MS/MS. Proteins identified in most of the groups (and their corresponding spot numbers) were tropomyosin (9), actins (10,12), SBP1 (14), Prx6 (17,18), CR (19), annexin (20), and SOD1 (21). Other proteins identified in cytosols from Group III mice were serpins (1,2,11), vinculin (3), albumin, (4,6-8), keratin (5), GRP 58 (13), hsc 70 (15), Rho GDP-dissociation inhibitor (16), and hemoglobin (22-24). (B) Cytosol from untreated mice. Spots correspond to albumin (1), unknown (2), and BHT-adducted trypsin inhibitor added as a positive control (described in reference 13).

Figure 3.

Analyses of human Prx6 and bovine SOD1 adducts formed with BHT-QM *in vitro*. (A) Coomassie-stained SDS-PAGE gels of human Prx6 and (B) the corresponding western blots. Proteins were treated with 0 (lane 1), 1 (lane 2), 5 (lane 3), or 10 (lane 4) molar equivalents of BHT-QM. Molecular weight standards are in lane 5 of each gel, and apparent minor differences in the positions of untreated relative to treated proteins are artifacts of these gels not due to inherent differences in their motilities.

Figure 4.

Effects of QMs on the activities of antioxidant enzymes. (A) Bovine Prx6 activity after incubations with 75 μ M BHT-QM for the time periods shown. (B) Prx6 activity after 30-min incubations with three concentrations of BHT-QM (●) or BHTOH-QM (■). (C) Activity of SOD1 after 30-min incubations with 0, 50, or 200 μ M BHT (●) or BHT-QM (○). Enzyme activities were measured as described in the Methods section.

Figure 5.

LC-MS/MS analysis of a tryptic digest of BHT-QM-treated human Prx6. (A) Base peak chromatogram with the adduct peaks indicated. (B) MS of peaks I and II containing doubly charged ions corresponding to adducts with MH^+ 1744.8 and 1556.8 Da, respectively. Peptide I underwent small degree of in-source fragmentation forming the BHT cation at m/z 219. CID analysis of each peptide adduct resulted in the loss of 218 Da yielding MS^2 spectra of the non-adducted peptides DINAYNCEEPTEK (MH^+ 1525.6) and DFTPVCTTELGR (MH^+ 1338.6), respectively. Peptide structures were confirmed by MS^3 analysis with the formation of y and b ions as shown.

Figure 6.

MALDI-TOF analysis of bovine apo-SOD1 after treatment with BHT-QM. (A) The intact protein mixture contained SOD1 with 0, 1, or 2 QM groups attached and average masses of 15592, 15811, and 16031 Da, respectively. A spectrum of untreated protein contained only the first of these three peaks, also at 15592 Da, and external mass calibration was carried out with apomyoglobin. (B) An Asp-N digest of adducted SOD1 showing the mass region containing an adduct ion at 1729.78 Da corresponding to the peptide shown. (C) List of all the peptides identified in the Asp-N digest highlighting two Cys residues and His 41 and 78 discussed in the text.

Table 1
Treatment Regimens for BALB Mice receiving BHT Injections.^a

Group:	I	II	III	IV	V	VI
injected on day(s): ^b	0	0	0	0, 7	0, 7, 14	0, 7, 14, 21
euthanized on day:	1 ^c	3	7	14	21	28

^a Each group consisted of 5 mice receiving 150 mg/kg of BHT ip.

^b Day 0 refers to time 0 (i.e., initiation of the experiment).

^c Group I mice were euthanized 24 h after dosing.

Table 2
Protein Adducts Formed in the Lungs of BHT-Injected Mice.^a

protein name	Groups ^b	Mr (kDa)	accession number	Matches ^c	coverage	Mowse score
SOD1	II, III, V, VI	15,802	NP_035564	11	56	438
Ptx6	I–VI	24,724	NP_041379	14	63	524
CR	I–VI	25,682	BAA05120	17	36	484
SBP1	II, III, V, VI	52,480	NP_033176	19	46	854
tropomyosin 5	I–VI	29,003	I11212	18	43	530
apolipoprotein A1	I–IV, VI	30,597	NP_033822	13	36	553
annexin A3	I–VI	36,334	NP_038498	14	41	784
β-actin	II, III, V, VI	39, 161	CAA27396	25	30	313

^a Only proteins determined to be adducted in at least four of the treatment groups are listed.
^b Adduct identified in the groups defined in Table 1.
^c Values are typical of the 4–6 determinations for each protein.

Table 3

Lipid Peroxidation Products, Superoxide, and Hydrogen Peroxide in S9 Fractions from Mouse Lungs Treated with BHT-QM *in vitro*.

treatment ^a	fold increase relative to control ^b		
	TBARS	O ₂ ⁻	H ₂ O ₂
untreated control	44 ± 3	0.22 ± 0.05	0.15 ± 0.01
1.0 mM TBHP	247 ± 4 (5.6)	nd ^c	nd
50 μM BHT-QM	166 ± 8 (3.8)	0.60 ± 0.06 (2.7)	0.46 ± 0.02 (3.0)
100 μM BHT-QM	292 ± 13 ^d (6.6)	0.66 ± 0.09 (3.0)	0.67 ± 0.09 (4.3)
200 μM BHT-QM	484 ± 8 (11)	nd	0.82 ± 0.13 (5.3)

^aIncubations were conducted at 37 °C for 30 min and controls contained MeCN only. Each result is the mean ± SD of 3 or 4 determinations.

^bUnits are nmol mg⁻¹ for TBARS, nmol mg⁻¹ min⁻¹ for O₂⁻, and mM for H₂O₂.

^cNot determined.

^dIncubation with 2 mM diethyl maleate for 30 min prior to adding 100 μM BHT-QM produced 383 ± 15 nmol TBARS mg⁻¹ protein

Table 4

Effects of Various Treatments on the Activity of Prx6.

treatment ^a	activity (nmol min ⁻¹ mg ⁻¹)
none	1377 ± 28
vehicle only	1340 ± 62
50 μM BHT	1364 ± 84
50 μM BHT-QM	737 ± 35
50 μM BHT-QM + dialysis	723 ± 82
50 μM BHT-QM + N-acetylcysteine ^b	1311 ± 83

^aIncubations were conducted with human Prx6 as described under Methods. BHT-QM was delivered in MeCN (solvent concentration of 2% v/v).

^bA 10-fold excess of N-acetylcysteine was added (relative to the QM).