Biochemical Pharmacology, Vol. 56, pp. 361–370, 1998.
© 1998 Elsevier Science Inc. All rights reserved.

ELSEVIER

ISSN 0006-2952/98/$19.00 + 0.00
PII S006-2952(98)00037-9

# Oxidative DNA Damage and Apoptosis Induced by Metabolites of Butylated Hydroxytoluene

*Saeko Oikawa,* Kohsuke Nishino,† Shinji Oikawa,* Sumiko Inoue,* Tamio Mizutani‡ and Shosuke Kawanishi*§*

*Department of Hygiene, Mie University School of Medicine, Mie 514; †Department of Food Science and Nutrition, Faculty of Human Life and Science, Doshisha Women's College, Kyoto 602; and ‡Department of Food Science, Kyoto Prefecture University, Kyoto 606, Japan

**ABSTRACT.** DNA damage by metabolites of a food additive, butylated hydroxytoluene (BHT), was investigated as a potential mechanism of carcinogenicity. The mechanism of DNA damage by 2,6-di-*tert*-butyl-*p*-benzoquinone (BHT-quinone), 2,6-di-*tert*-butyl-4-hydroperoxyl-4-methyl-2,5-cyclohexadienone (BHT-OOH), and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-CHO) in the presence of metal ions was investigated by using $^{32}$P-labeled DNA fragments obtained from the c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. BHT-OOH caused DNA damage in the presence of Cu(II), whereas BHT-quinone and BHT-CHO did not. However, BHT-quinone did induce DNA damage in the presence of NADH and Cu(II). Bathocuproine inhibited Cu(II)-mediated DNA damage, indicating the participation of Cu(I) in the process. Catalase also inhibited DNA damage induced by BHT-quinone, but not that induced by BHT-OOH. The DNA cleavage pattern observed with BHT-quinone plus NADH was different from that seen with BHT-OOH. With BHT-quinone plus NADH, piperidine-labile sites could be generated at nucleotides other than adenine residue. BHT-OOH caused cleavage specifically at guanine residues. Pulsed field gel electrophoresis showed that BHT-OOH and BHT-quinone induced DNA strand breaks in cultured cells, whereas BHT-CHO did not. Both BHT-quinone and BHT-OOH induced internucleosomal DNA fragmentation, which is the characteristic of apoptosis. Furthermore, flow cytometry analysis revealed an increase of peroxides in cultured cells treated with BHT-OOH or BHT-quinone. These results suggest that BHT-OOH participates in oxidative DNA damage directly, whereas BHT-quinone causes DNA damage through $H_2O_2$ generation, which leads to internucleosomal DNA fragmentation. BIOCHEM PHARMACOL **56**;3:361–370, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** DNA damage; apoptosis; BHT; copper; hydrogen peroxides

BHT‖ is a synthetic phenolic antioxidant widely used as a food additive. It has been reported that BHT increases the incidence of hepatocellular tumors in male mice [1]. BHT has been suggested to be carcinogenic in the liver of rats [2]. In addition, BHT has tumor-promoting activity when given after an initiating carcinogen in the mouse lung [3] and colon [4], and in the rat liver [5, 6] and urinary bladder [7]. On the other hand, BHT has been shown to inhibit the induction of cancer by a wide variety of chemical carcinogens [8]. It is noteworthy that BHT has adverse effects in enhancing and preventing tumor development in a variety of tissues and organs. BHT has not been shown to be mutagenic in Salmonella test systems [9, 10].

Studies on the metabolism of BHT *in vivo* and *in vitro* have revealed that there are two main metabolic processes in the mouse and rat, hydroxylation of the alkyl substituents and oxidation of the aromatic ring system. BHT-COOH is a major metabolite formed from the former process and may be derived via the corresponding alcohol and aldehyde (BHT-CHO) [11]. BHT-quinone has been identified as a further metabolite of BHT-COOH [12]. Moreover, recent studies have demonstrated the occurrence of a series of metabolites arising from oxidation of the *tert*-butyl groups [13]. Oxidation of the aromatic system leads to the formation of a number of minor metabolites such as BHT-OOH [11] and 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (BHT-quinone methide) [14]. It has been reported that certain metabolites of BHT induce strand breaks in plasmid DNA [15, 16]. However, the mechanism remains to be clarified. In this study, we investigated the mechanism of DNA damage by BHT-OOH, BHT-quinone, and BHT-CHO in the presence of NADH and endogenous metal ions using $^{32}$P 5′ end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 proto-oncogene.

The relationships between DNA damage, apoptosis, and

§ Corresponding author: Dr. Shosuke Kawanishi, Department of Hygiene, Mie University School of Medicine, Mie 514, Japan. FAX +81-592-31-5011; E-mail: kawanisi@doc.medic.mie-u.ac.jp.

‖ *Abbreviations:* BHT, butylated hydroxytoluene; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-quinone, 2,6-di-*tert*-butyl-*p*-benzoquinone; BHT-OOH, 2,6-Di-*tert*-butyl-4-hydroperoxyl-4-methyl-2,5-cyclohexadienone; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; DCFH-DA, 2′, 7′-dichlorofluorescin diacetate; DTPA, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid; $H_2O_2$, hydrogen peroxide; ·OH, hydroxyl free radical; and $O_2^-$, superoxide anion radical.

Received 24 June 1997; accepted 7 January 1998.

carcinogenesis are of considerable interest. Apoptosis contributes to the pathogenesis of a number of diseases, including cancer [17, 18]. Apoptosis is induced by many cytotoxic chemicals and ionizing radiation, and is characterized by morphological and biochemical changes, such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, and DNA fragmentation at internucleosomal sites. Although BHT has been shown to induce necrosis in mice [19], there are no reports concerning BHT-induced apoptosis. To improve the understanding of BHT carcinogenicity, we investigated cellular DNA damage induced by various BHT metabolites. DNA strand breaks in human cultured cells were investigated by pulsed field gel electrophoresis. The relationship between cellular DNA damage and formation of the DNA "ladder," which is characteristic of apoptosis, was examined. In addition, intracellular peroxide levels in cells treated with BHT metabolites were analyzed by flow cytometry.

## MATERIALS AND METHODS

### *Materials*

Restriction enzymes (*Bam*HI, *Ava*I, *Xba*I, *Pst*I and *Hind*III) and $T_4$ polynucleotide kinase were purchased from New England Biolabs. Restriction enzymes, *Eco*RI, *Sma*I, *Apa*I and *Sty*I, were from Boehringer Mannheim GmbH. A human *p53* amplimer panel was from Clontech Lab. The primers designed for the PCR process for the amplification of *p53* are contained in this product (kit). [$\gamma$-$^{32}$P]ATP (222 TBq/mmol) was from New England Nuclear. DTPA and bathocuproinedisulfonic acid were from Dojindo Laboratories. Acrylamide, DMSO, bisacrylamide and piperidine were from Wako Pure Chemical Industries. $CuCl_2$, ethanol, D-mannitol, and sodium formate were from Nacalai Tesque, Inc. Catalase (45,000 U/mg from bovine liver) was from Sigma. BHT-quinone was from Tokyo Kasei Organic Chemicals. BHT-OOH and BHT-CHO were synthesized according to the methods described by Kharasch and Joshi [20] and Coppinger and Campbell [21], respectively. DNA fragments were obtained from human *p53* tumor suppressor gene [22] and c-Ha-*ras*-1 proto-oncogene [23].

### *Subcloning of* EcoRI-HindIII *Fragments from* p53 *Tumor Suppressor Gene*

Two fragments from *p53* gene containing exons were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with *Sma*I and ligated into *Sma*I-cleaved pUC 18 plasmid, and then transferred to *Escherichia coli* JM 109.

### *Preparation of $^{32}$P 5′ End-Labeled DNA Fragments Obtained from* p53 *Tumor Suppressor Gene*

The plasmid pUC 18 was digested with *Eco*RI and *Hind*III, and the resulting DNA fragments were fractionated by electrophoresis on 2% agarose gel. A 5′ end-labeled 654-base pair fragment (*Hind*III*13972–*Eco*RI*14621) and a 461-base pair fragment (*Hind*III*13038–*Eco*RI*13507) were obtained by dephosphorylating with calf intestine phosphatase and rephosphorylating with [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase. The 654-base pair fragment was further digested with *Apa*I to obtain a singly labeled 443-base pair fragment (*Eco*RI*14621–*Apa*I 14179) and a 211-base pair fragment (*Apa*I 14182–*Hind*III*13972). The 461-base pair fragment was further digested with *Sty*I to obtain a singly labeled 343-base pair fragment (*Sty*I 13160–*Eco*RI*13507) and a 118-base pair fragment (*Hind*III*13038–*Sty*I 13155). The asterisk indicates $^{32}$P-labeling.

### *Preparation of $^{32}$P 5′ End-Labeled DNA Fragments Obtained from c-Ha-*ras*-1 Proto-oncogene*

DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 proto-oncogene [24, 25]. A singly labeled 98-base pair fragment (*Ava*I* 2247–*Pst*I 2344) and 337-base pair fragment (*Pst*I 2345–*Ava*I* 2681) were obtained according to the method described previously [24, 25]. The asterisk indicates $^{32}$P-labeling and nucleotide numbering starts at the *Bam*HI site [23].

### *Detection of Damage to Isolated DNA Induced by BHT Metabolites in the Presence of Metal Ions*

The standard reaction mixture in a microtube (1.5-mL Eppendorf) contained various concentrations of BHT metabolites (BHT-quinone, BHT-OOH, and BHT-CHO), 250 $\mu$M NADH, 20 $\mu$M $CuCl_2$, [$^{32}$P]DNA fragment, and 1 $\mu$M per base of sonicated calf thymus DNA in 200 $\mu$L of 10 mM sodium phosphate buffer (pH 7.8) containing 5 $\mu$M DTPA. After incubation at 37° for 120 min, the DNA fragments were heated at 90° in 1 M piperidine for 20 min where indicated and treated as previously described [24, 25]. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [26] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

### *Cell Lines*

Burkitt's lymphoma cell line BJAB and human myelogenous leukemic cell line HL 60 were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 5% fetal calf serum (FCS) (Whittaker Bioproducts) at 37° under 5% $CO_2$ in a humidified atmosphere. Cells ($10^6$ cells/mL) were incubated for 4 hr or 17 hr with or without the BHT metabolites. Cells were preincubated with $ZnSO_4$ for 1 hr, where indicated.

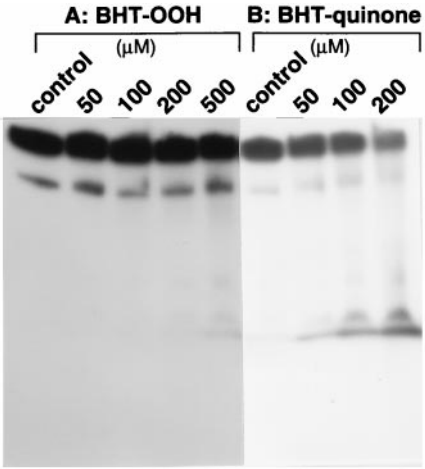


**FIG. 1. Autoradiogram of $^{32}$P-labeled DNA fragments incubated with BHT-OOH and BHT-quinone plus NADH in the presence of Cu(II). The reaction mixture contained the $^{32}$P 5′ end-labeled 98-base pair fragment (*Ava*I* 2247–*Pst*I 2344) from c-Ha-*ras*-1 DNA fragment, 1 μM per base of sonicated calf thymus DNA, BHT metabolite ((A) BHT-OOH or (B) BHT-quinone + 250 μM NADH) and 20 μM $CuCl_2$ in 200 μL of 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated at 37° for 120 min. The DNA fragments were treated with 1 M piperidine for 20 min at 90°, and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing x-ray film to the gel.**

### *Detection of Cellular DNA Damage by Pulsed Field Gel Electrophoresis*

For the determination of DNA strand breaks, the treated cells were washed twice with RPMI 1640 and once with PBS and resuspended in 150 μL of PBS. The cell suspension was solidified with agarose, followed by incubation with proteinase K according to the method described previously [27]. Electrophoresis was performed in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) by the pulsed field electrophoresis system (CHEF-DRII DNA megabase electrophoresis system, Bio-Rad) at 200 volts at 14°. The switch time was 60 sec for 15 hr followed by a 90 sec switch time for 9 hr. The DNA in the gel was visualized using ethidium bromide.

### *Detection of DNA "Ladder" Formation*

After the treated cells were washed, as described for pulsed field gel electrophoresis, the cells were disrupted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100. Nuclei were pelleted by centrifugation at 1000 *g*. The nuclei were then extracted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.4 M NaCl, 1 mM EDTA, and 1% Triton X-100 and centrifuged at 12,000 *g* to separate the nucleoplasm from the high molecular weight chromatin. Nucleoplasm was incubated with RNase (final concentration 20 μg/mL) for 1 hr and then with proteinase K (final concentration 100 μg/mL) for 2 hr at 37°. DNA was purified with phenol-chloroform and precipitated with 2.5 vol of ethanol and analyzed by conventional electrophoresis on a 1.4% agarose gel containing 0.5 mg of ethidium bromide per liter at 50 volts for 2 hr.

### *Flow-Cytometric Detection of Organic Peroxides and $H_2O_2$ in Cultured Cells Treated with BHT Metabolites*

HL 60 cells ($1 \times 10^6$ cells/mL) were incubated with either BHT-OOH or BHT-quinone in RPMI 1640 containing 6% FCS for 3.5 hr at 37°. Five μM DCFH-DA, a sensitive fluorometric probe for peroxides [28, 29], was added to the medium before incubating the cells for 30 min at 37°. After the incubation, the medium was removed and the cells were washed with PBS once and suspended in 2 mL of PBS. The cells were analyzed with a FACScan (Becton Dickinson).

## RESULTS

### *Damage to $^{32}$P-Labeled DNA Fragments Induced by BHT Metabolites in the Presence of Cu(II)*

Figure 1A shows the autoradiogram of DNA fragments treated with BHT-OOH in the presence of Cu(II). DNA cleavage increased with the concentration of BHT-OOH and with time. The upper and lower bands in the control show single-stranded and double-stranded forms of the

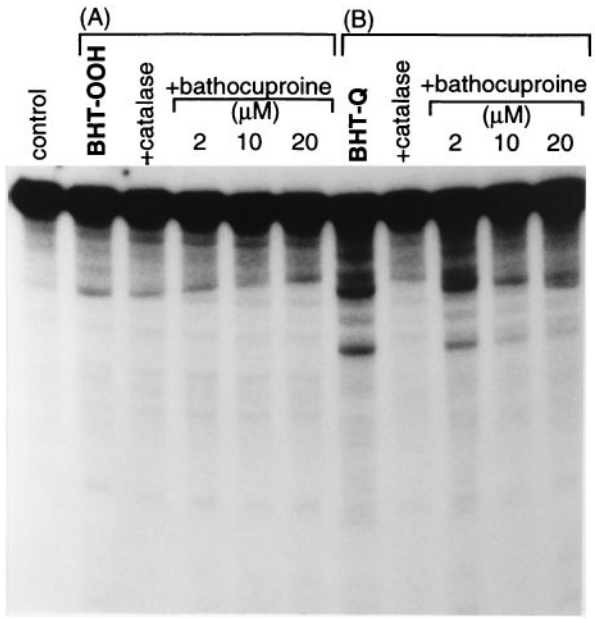


**FIG. 2. Effects of catalase and bathocuproine on DNA cleavage induced by BHT-OOH and BHT-quinone plus NADH in the presence of Cu(II). The $^{32}$P 5′ end-labeled 337-base pair fragment (*Pst*I 2345–*Ava*I* 2681) and 1 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.8 containing 5 μM DTPA were incubated with 2 mM BHT-OOH plus 20 μM $CuCl_2$ (A) or 2 mM BHT-quinone plus 20 μM $CuCl_2$ in the presence of 100 μM NADH (B). Thirty units of catalase or bathocuproine was added where indicated. After the incubation at 37° for 120 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the Fig. 1 legend.**

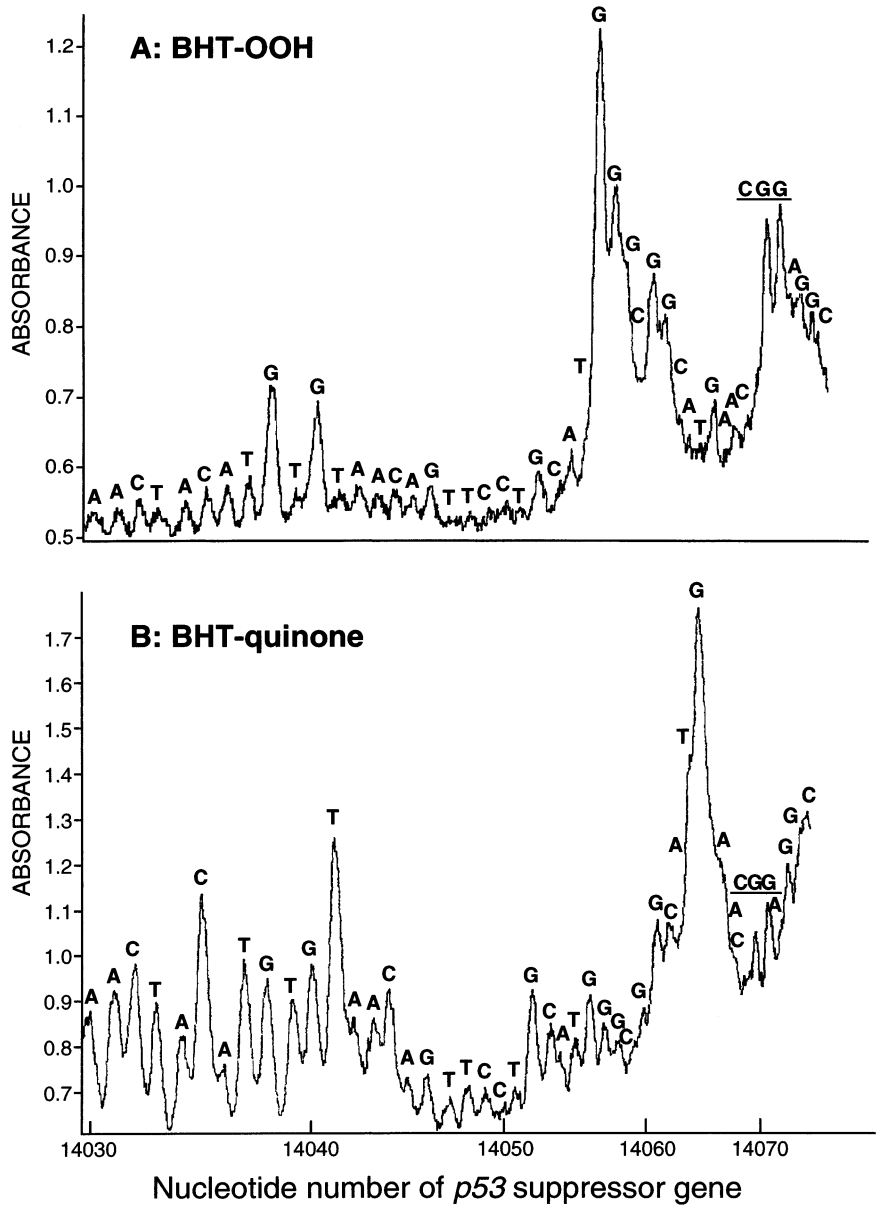


**FIG. 3. Site specificity of DNA cleavage by BHT-OOH and BHT-quinone plus NADH in the presence of Cu(II). The $^{32}$P 5′ end-labeled 211-base pair fragment (*Apa*I 14182–*Hind*III*13972) from *p53* in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 1 μM per base of sonicated calf thymus DNA was incubated with BHT metabolite ((A) 1 mM BHT-OOH or (B) 1 mM BHT-quinone + 100 μM NADH) at 37° for 120 min. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide/8 M of urea gel using a DNA-sequencing system, and the autoradiogram was obtained by exposing x-ray film to the gel. The relative amounts of oligonucleotides produced were measured by a laser densitometer (LKB 2222 UltroScan XL). The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after undergoing DNA sequence reaction according to the Maxam-Gilbert procedure [26]. The horizontal axis shows the nucleotide number of human *p53* tumor suppressor gene [22]. Underscoring indicates the hot spot of exon 8 of human *p53* tumor suppressor gene.**

intact DNA fragment, respectively. Neither BHT-OOH nor Cu(II) caused DNA damage. BHT-OOH did not cause DNA damage in the presence of Fe(III)EDTA. BHT-CHO did not induce DNA damage in the presence of Cu(II) (data not shown).

Although BHT-quinone did not induce DNA damage in the presence of Cu(II) alone, BHT-quinone did induce Cu(II)-dependent DNA damage in the presence of NADH. DNA damage in the presence of Cu(II) and NADH increased with increasing concentrations of BHT-quinone (Fig. 1B). DNA damage was enhanced by piperidine treatment, suggesting that BHT-quinone plus NADH caused not only DNA strand breakage but also base modification and/or liberation. Damage to denatured single-stranded DNA was stronger than that to native double-stranded DNA. At the concentration used (250 μM), NADH did not induce Cu(II)-dependent DNA damage in the absence of BHT-quinone. Relevantly, we have reported previously that in the presence of Cu(II), DNA damage is caused by more than NADH present at 1-mM concentration [30]. BHT-quinone plus NADH did not cause DNA damage in the presence of Fe(III)EDTA (data not shown).

The effects of catalase and bathocuproine on Cu(II)-mediated DNA damage by BHT-quinone plus NADH and BHT-OOH were then investigated. Catalase completely inhibited Cu(II)-mediated DNA damage by BHT-quinone (Fig. 2B), suggesting the involvement of $H_2O_2$. In contrast, catalase did not inhibit Cu(II)-mediated DNA damage by BHT-OOH (Fig. 2A). The addition of 10 μM of bathocuproine inhibited Cu(II)-mediated DNA damage by BHT-quinone plus NADH, suggesting the involvement of Cu(I) (Fig. 2B). Although 10 μM bathocuproine did not inhibit Cu(II)-mediated DNA damage by BHT-OOH (Fig. 2A), the addition of DTPA inhibited Cu(II)-mediated DNA

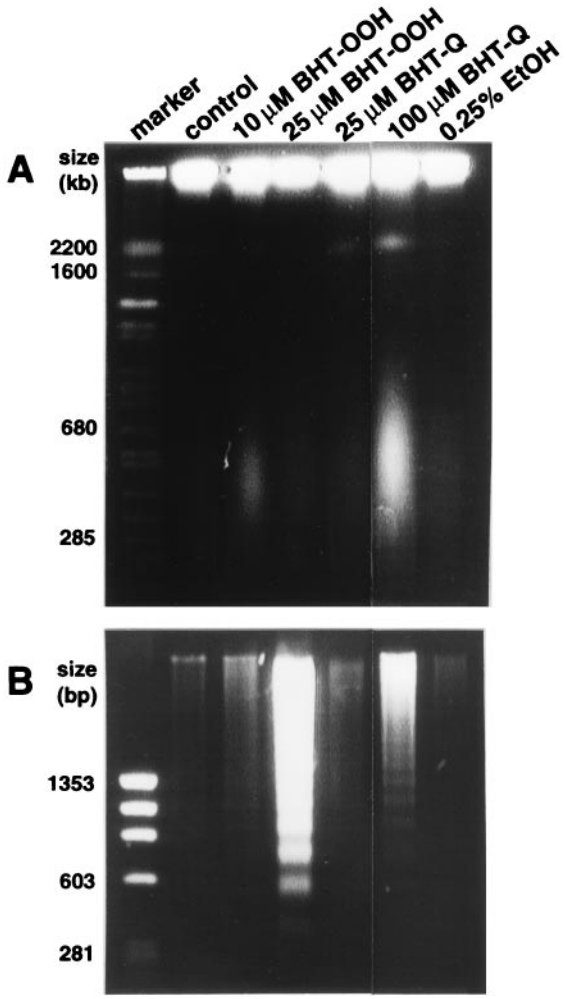


**FIG. 4. Detection of cellular DNA damage and DNA ladder formation in cultured cells treated with BHT metabolites. HL 60 cells were treated with either BHT-OOH or BHT-quinone for 4 hr at 37°. (A) Cells were prepared as agarose plugs, lysed and subjected to pulsed field gel electrophoresis through 1% agarose gel, as described in Materials and Methods. The gel was stained in ethidium bromide. Marker lane: size marker DNA (*Saccharomyces cerevisiae*). (B) The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Materials and Methods. Marker lane: size marker DNA (ϕX 174/*Hae*III digest). BHT-Q indicates BHT-quinone.**

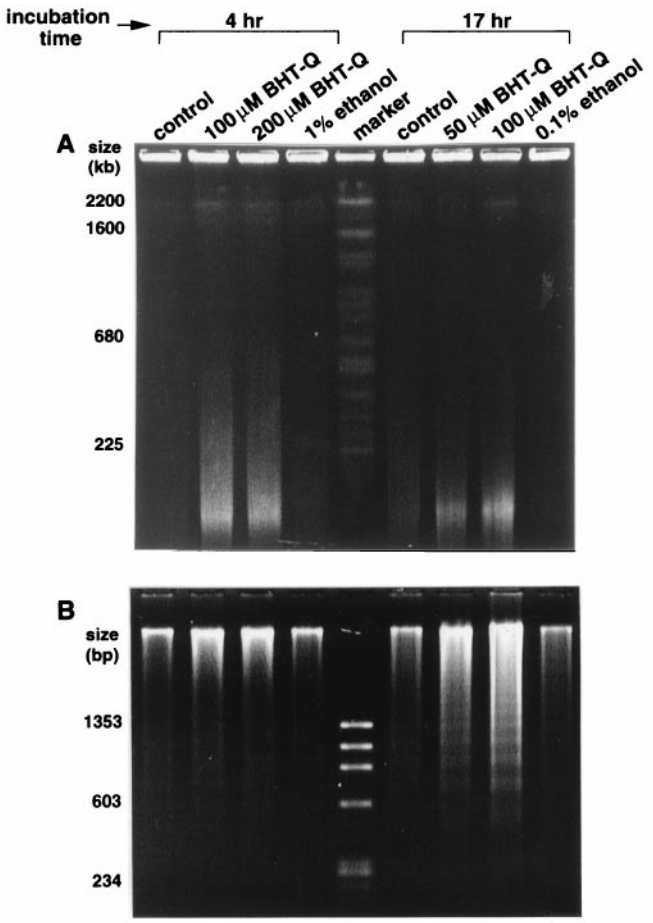


**FIG. 5. BHT-quinone-induced cellular DNA damage and subsequent DNA ladder formation. BJAB cells were treated with BHT-quinone for 4 hr and 17 hr at 37°. (A) The cells were prepared into agarose plugs, lysed and subjected to pulsed field gel electrophoresis through 1% agarose gel, as described in Materials and Methods. The gel was stained in ethidium bromide. Marker lane, size marker DNA (*Saccharomyces cerevisiae*). (B) The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Materials and Methods. Marker lane, size marker DNA (ϕX 174/*Hae*III digest). BHT-Q indicates BHT-quinone.**

damage, suggesting the involvement of Cu(II) binding to DNA. Typical hydroxyl radical (●OH) scavengers showed no inhibitory effects on Cu(II)-mediated DNA damage by these compounds (data not shown).

### *Site Specificity of Cu(II)-mediated DNA Damage Induced by BHT-OOH and BHT-Quinone Plus NADH*

To investigate any site specificity of Cu(II)-mediated DNA cleavage, $^{32}$P 5′ end-labeled DNA fragments incubated with either BHT-OOH or BHT-quinone plus NADH in the presence of Cu(II), followed by piperidine treatment, were electrophoresed and the autoradiograms scanned with a laser densitometer (Fig. 3). When DNA was incubated with BHT-OOH plus Cu(II), cleavage occurred specifically at guanine residues. Cleavage at or near the hot spot in exon 8 of the *p53* tumor suppressor gene was comparatively strong under the present conditions (Fig. 3A). DNA cleavage was not observed without piperidine treatment. The results suggest that the formation of direct strand breaks is far less important than the induction of piperidine-labile sites at guanine bases in the case of the mixture of BHT-OOH and Cu(II).

When BHT-quinone plus NADH was used instead of BHT-OOH, the site specificity of Cu(II)-mediated DNA cleavage was changed. Cytosine, thymine and guanine residues were piperidine-labile sites. Adenine residue was relatively resistive to piperidine treatment (Fig. 3B), although piperidine-labile sites are only representative of a broad class of oxidative damage to DNA including a few base lesions and sugar modifications. The site specificity

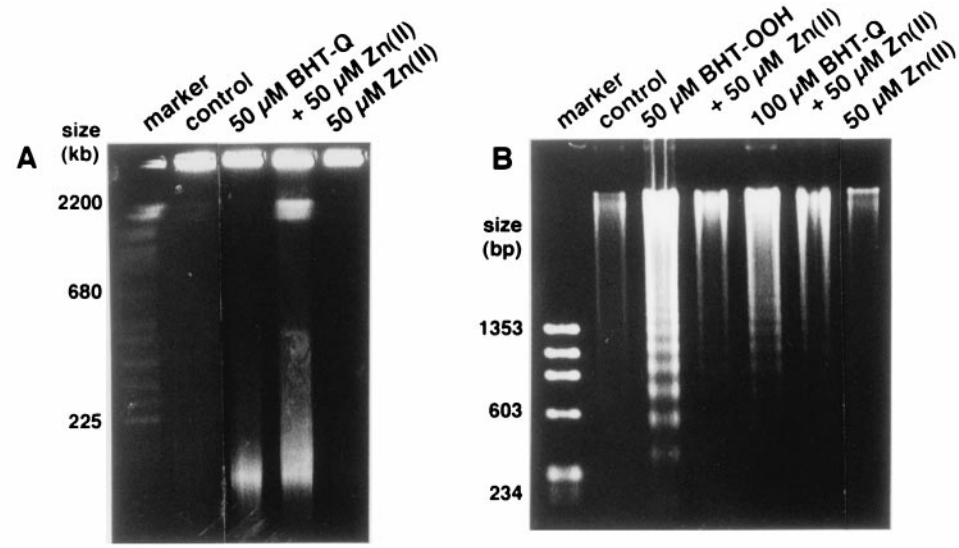


**FIG. 6. Effect of Zn(II) on BHT metabolite-induced cellular DNA damage and DNA ladder formation. (A) BJAB cells were treated with BHT-quinone for 17 hr at 37°. The cells were prepared into agarose plugs, lysed and subjected to pulsed field gel electrophoresis on 1% agarose gel, as described in Materials and Methods. The gel was stained in ethidium bromide. Marker lane, size marker DNA (*Saccharomyces cerevisiae*). (B) BJAB cells were treated with BHT-OOH or BHT-quinone for 4 hr at 37°. The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Materials and Methods. Marker lane, size marker DNA (ϕX 174/*Hae*III digest). BHT-Q indicates BHT-quinone.**

was similar to that of DNA cleavage induced by high concentrations of NADH in the presence of Cu(II) (data not shown) [30].

### *Cellular DNA Damage and Internucleosomal DNA Fragmentation Induction by BHT Metabolites*

We analyzed DNA strand breaks in cells treated with BHT metabolites using pulsed field gel electrophoresis. DNA fragments corresponding to 500–600 kb were observed in HL 60 cells incubated with 10 μM BHT-OOH, and DNA fragments corresponding to *ca.* 2 Mb and 500–600 kb were observed with 100 μM BHT-quinone (Fig. 4A). Agarose gel electrophoresis of DNA extracted from HL 60 cells treated with 25 μM BHT-OOH and 100 μM BHT-quinone revealed a characteristic DNA ladder pattern, which is associated with apoptosis (Fig. 4B). BHT-OOH induced stronger DNA ladder formation than BHT-quinone.

Figure 5 shows that treatment of BJAB cells with BHT-quinone for 4 hr caused DNA cleavage, producing DNA fragments of *ca.* 2 Mb and 50 kb, but not DNA ladder formation. Treatment for 17 hr induced not only DNA cleavage at 50 kb (Fig. 5A) but also DNA ladder formation (Fig. 5B). These results suggest that BHT metabolites cause DNA damage in cells, resulting in internucleosomal DNA fragmentation and apoptosis.

Zn(II) inhibited BHT metabolite-mediated DNA ladder formation (Fig. 6B). However, formation of the *ca.* 2-Mb DNA fragments increased upon addition of Zn(II) (Fig. 6A). These results indicate that an endonuclease participates in DNA ladder formation. Therefore, it has been reasonably speculated that BHT metabolites cause oxidative DNA damage to cleavage into *ca.* 2-Mb fragments, followed by enzymatic cleavage to smaller DNA fragments.

### *Detection of Intracellular Peroxides Induced by BHT Metabolites*

Figure 7 shows flow-cytometric distribution of HL 60 cells treated with BHT metabolites, and subsequently with DCFH-DA, a probe to detect intracellular peroxide production. Treatment with 10 μM BHT-OOH and 50 μM BHT-quinone resulted in an increase in fluorescence intensity, suggesting that intracellular peroxides were increased by BHT metabolites. In the case of BJAB cells, BHT-OOH increased in fluorescence intensity more efficiently than BHT-quinone (data not shown).

## DISCUSSION

The present results have shown that BHT-OOH caused damage to isolated DNA in the presence of Cu(II), whereas BHT-quinone and BHT-CHO did not. However, the addition of NADH induced DNA damage by BHT-quinone in the presence of Cu(II). The metal-mediated DNA cleavage pattern observed with BHT-OOH was different from that with BHT-quinone and NADH. BHT-OOH plus Cu(II) induced specific oxidation of guanine residues, and subsequent piperidine treatment led to chain cleavage at each guanine residue. On the other hand, in the presence of Cu(II), BHT-quinone plus NADH frequently induced piperidine-labile sites at cytosine, thymine and guanine residues, but rarely at adenine residue. In other words, piperidine-labile sites were guanine residues and pyrimidine residues especially located 5′ and/or 3′ to purine residues. In order to examine what kinds of active species caused the two types of site-specific DNA damage, experiments using various scavengers were performed. Typical ·OH scavengers showed no inhibitory effect on Cu(II)-mediated DNA damage. Bathocuproine inhibited DNA damage, indicating the participation of Cu(I). Catalase inhibited DNA damage by BHT-quinone, but not that by BHT-OOH. These results

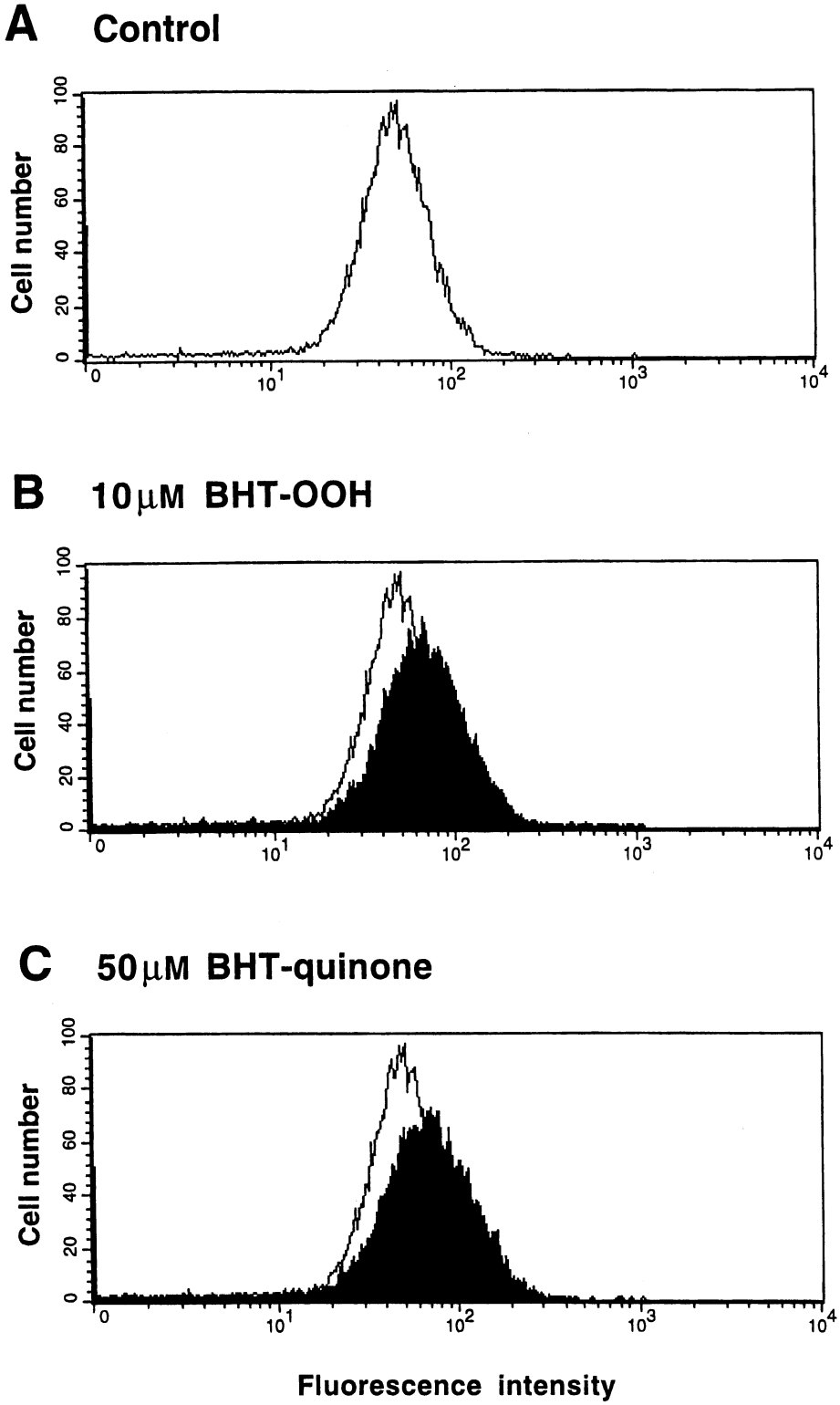


**FIG. 7. Flow-cytometric fluorescence distributions of cultured cells treated with BHT metabolites. HL 60 cells were treated with either BHT-OOH or BHT-quinone for 4 hr at 37°, and 5 μM DCFH-DA was added to medium followed by incubation for 30 min at 37°. The cells were analyzed with a flow cytometer (FACScan). (A) Control cells treated with 0.05% ethanol. (B) Cells treated with 10 μM BHT-OOH. (C) Cells treated with 50 μM BHT-quinone. The horizontal axis shows the relative fluorescence intensity, and the vertical axis shows the cell number. Distributions of fluorescence intensity of control are shown in lines B and C.**

suggest that an active complex derived from $H_2O_2$ and Cu(I) rather than ·OH may play an important role in metal-dependent DNA damage by BHT-quinone and NADH. This complex may be a bound hydroxyl radical, which immediately attacks an adjacent DNA constituent before being intercepted by ·OH scavengers. In contrast, BHT-OOH itself participates in DNA damage. It has previously been reported that BHT-OOH can be converted into phenoxyl and alkoxyl radicals [31, 32]. It is considered that this alkoxyl radical may be the ultimate reactive species in guanine-specific DNA damage, because it has been reported that similar site-specific cleavage can be

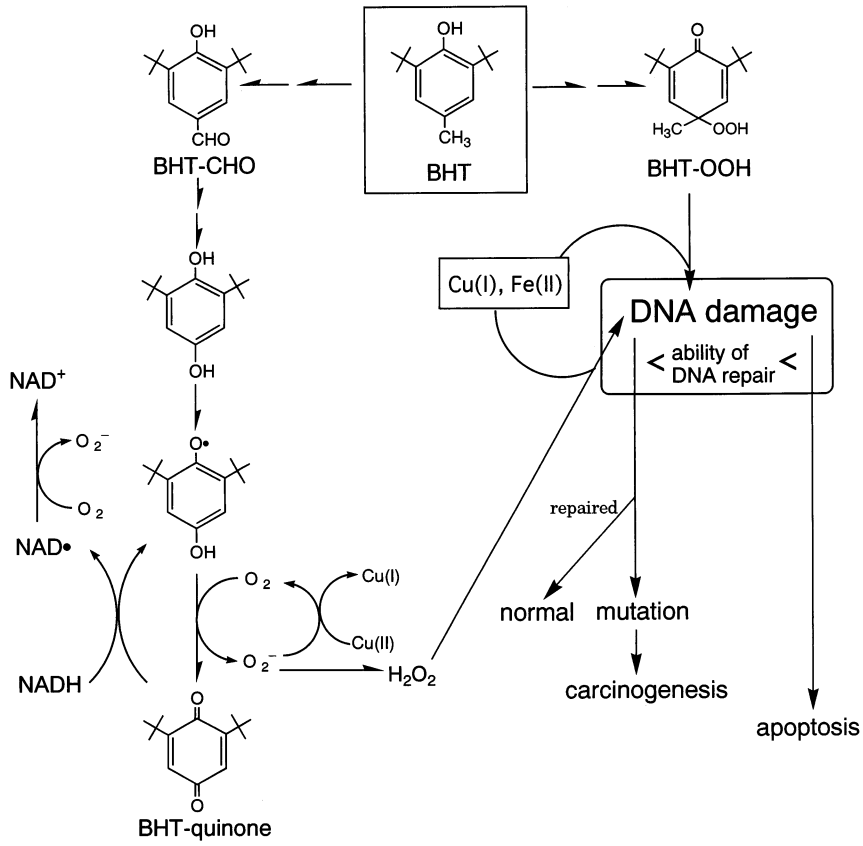


**FIG. 8. Possible mechanisms for DNA damage and apoptosis induced by BHT metabolites.**

induced by hydroperoxide of linoleic acid which may produce the corresponding alkoxyl radical [33]. The site specificity by BHT-OOH cannot be explained by ·OH, because it is generally considered that ·OH causes DNA cleavage at every nucleotide with no marked site specificity when ·OH is produced by a metal complex not bound to DNA or by γ-radiolysis of $H_2O$ [24, 25, 34, 35]. It is known that the energy level of the highest occupied molecular orbital of guanine is highest among the nucleic acid bases and, accordingly, guanine is oxidized most easily [36]. The predominant guanine alteration in BHT-OOH-induced DNA damage can be explained by the fact that alkoxyl radical has less oxidizing potential than ·OH.

The pulsed field gel electrophoresis experiments showed that treatment with BHT-OOH and BHT-quinone for 4 hr produced strand breaks in DNA of cultured human cells. Treatment with BHT metabolites for 17 hr caused not only DNA cleavage but also DNA ladder formation. Zn(II), a Ca(II)/Mg(II)-dependent endonuclease inhibitor, inhibited BHT metabolite-mediated DNA ladder formation (Fig. 6B). However, formation of *ca.* 2-Mb DNA fragments was not inhibited, but increased, by the addition of Zn(II). These results suggest that BHT metabolites cause oxidative DNA damage to cleave into *ca.* 2-Mb fragments, followed by enzymatic cleavage to smaller DNA fragments. Relevantly, Guyton *et al.* [37] reported that cell death induced by BHT-OOH was characterized by alterations in the mitochondrial and nuclear membranes.

On the basis of the above findings, possible mechanisms for DNA damage induced by BHT metabolites have been proposed as shown in Fig. 8. There are two pathways: one is via BHT-OOH and the second is via BHT-quinone. BHT-quinone generates $H_2O_2$ in the cell. The idea that $H_2O_2$ formation occurs via the mechanism shown in Fig. 8 may be supported by flow cytometry analysis and UV-visible spectroscopy. BHT-quinone is reduced by NADH to its semiquinone, which reacts with $O_2$ to produce $O_2^-$ and subsequently $H_2O_2$. Furthermore, formed NAD· also reacts with $O_2$ to form $NAD^+$ and $O_2^-$ at an almost diffusion-controlled rate [38]. Several other papers have also pointed out the possibility that chemicals are nonenzymatically reduced by NADH *in vivo* [39–41].

Flow cytometry analysis also supported the notion that BHT-OOH can penetrate into cells. Consequently, $H_2O_2$ and BHT-OOH reach into DNA. Then, $H_2O_2$ and BHT-OOH are activated by endogenous transition metal ions such as Cu(I) or Fe(II) to cause damage to cellular DNA. These active species cause damage to DNA and generate fragments corresponding to *ca.* 2 Mb, subsequently leading to smaller DNA fragments. Recently, it has been reported that $H_2O_2$ participates in endonuclease activation leading to apoptosis [42, 43].

The present work suggests that Cu(II) is an important factor in DNA damage induced by metabolites of BHT. Copper is naturally present in cell nuclei [44], and its role in carcinogenicity and mutagenicity has recently drawn

much interest [45–48]. The binding of copper to DNA and/or protein in chromatin is proposed to serve physiological functions [46], whereas copper bound to DNA and/or protein may provide an adventitious site for deleterious redox reactions [45]. Certain carcinogens may induce active oxygen species formation in the presence of endogenous metal ions and subsequently cause oxidative DNA damage [49]. Thus, the copper-mediated DNA damage by BHT metabolites seems to be relevant to the expression of the carcinogenicity of BHT.

The relationships between DNA damage, apoptosis, and carcinogenesis have attracted considerable interest. Cells that incur DNA damage and undergo apoptosis are no longer candidates for producing cancer cells. Moreover, the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a variety of human diseases, including cancer [17, 18]. DNA damage induces the production of the p53 protein, activation of proteases, and subsequent activation of endonucleases to catalyze DNA fragmentation at internucleosomal sites, leading to apoptosis [50]. We should also pay attention to errors in the repair of DNA damage that might cause the high mutation rate observed in many human cancers. The present study suggests that BHT metabolites generate peroxides and induce cellular DNA damage preceding apoptosis. This has led us to propose three fates of the cells with DNA damage, as shown in Fig. 8. Firstly, DNA damage is correctly repaired. Secondly, when the DNA damage is repaired incorrectly, the cells may undergo mutation, subsequently leading to cancer. Thirdly, stronger DNA damage, which fails to be repaired, may induce apoptotic cell death. The fates of the cells might be dependent on the intensity of DNA damage and the ability of DNA repair. Thus, it is proposed that various external stimuli resulting in apoptosis have genotoxic risk for cells.

*The authors are grateful to Dr. M. J. Burkitt for helpful suggestions in the preparation of this manuscript. This work was supported by Grants-in-Aid for Scientific Research No. 06557028 and No. 06454227 from the Ministry of Education, Science and Culture of Japan.*

## References

1. Inai K, Kobuke T, Nambu S, Takemoto T, Kou E, Nishina H, Fujihara M, Yonehara S, Suehiro S, Tsuya T, Horiuchi K and Tokuoka S, Hepatocellular tumorigenicity of butylated hydroxytoluene administered orally to B6C3F1 mice. *Jpn J Cancer Res* **79:** 49–58, 1988.
2. Olsen P, Meyer O, Bille N and Würtzen G, Carcinogenicity study on butylated hydroxytoluene (BHT) in Wister rats exposed *in utero*. *Food Chem Toxicol* **24:** 1–12, 1986.
3. Witschi H, Williamson D and Locks S, Enhancement of urethane tumorigenesis in mouse lungs by butylated hydroxytoluene. *J Natl Cancer Inst* **58:** 301–305, 1977.
4. Lindenschmidt RC, Tryka AF, Goad ME and Witschi HP, The effects of dietary butylated hydroxytoluene on liver and colon tumor development in mice. *Toxicology* **38:** 151–160, 1986.
5. Peraino C, Fry RJM, Staffeldt E and Christopher JP, Enhancing effects of phenobarbitone and butylated hydroxytoluene on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. *Food Cosmet Toxicol* **15:** 93–96, 1977.
6. Maeura Y. and Williams GM, Enhancing effect of butylated hydroxytoluene on the development of liver altered foci and neoplasms induced by *N*-2-fluorenylacetamide in rats. *Food Chem Toxicol* **22:** 191–198, 1984.
7. Imaida K, Fukushima S, Shirai T, Masui T, Ogiso T and Ito N, Promoting activities of butylated hydroxyanisole, butylated hydroxytoluene and sodium-L-ascorbate on forestomach and urinary bladder carcinogenesis initiated with the methylnitrosourea in F344 male rats. *Gann* **75:** 769–775, 1984.
8. Slaga TJ, Inhibition of skin tumor initiation, promotion, and progression by antioxidants and related compounds. *Crit Rev Food Sci Nutrit* **35:** 51–57, 1995.
9. Williams GM, McQueen CA and Tong C, Toxicity studies of butylated hydroxyanisole and butylated hydroxytoluene. I. Genetic and cellular effects. *Food Chem Toxicol* **28:** 793–798, 1990.
10. Bomhard EM, Bremmer JN and Herbold BA, Review of the mutagenicity/genotoxicity of butylated hydroxytoluene. *Mutat Res* **277:** 187–200, 1992.
11. Matuo M, Mihara K, Okuno M, Ohkawa H and Miyamoto J, Comparative metabolism of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) in mice and rat. *Food Chem Toxicol* **22:** 345–354, 1984.
12. Yamamoto K, Tajima K, Takemura M and Mizutani T, Further metabolism of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, a major metabolite of butylated hydroxytoluene in rats. *Chem Pharm Bull Tokyo* **39:** 512–514, 1991.
13. Thompson JA, Malkinson AM, Wand MD, Mastovich SL, Mead EW, Schullek KM and Laudenschlager WG, Oxidative metabolism of butylated hydroxytoluene by hepatic and pulmonary microsomes from rats and mice. *Drug Metab Dispos* **15:** 833–840, 1987.
14. Guyton KZ, Bhan P, Kuppusamy P, Zweier JL, Trush MA and Kensler TW, Free radical-derived quinone methide mediates skin tumor promotion by butylated hydroxytoluene hydroperoxide: expanded role for electrophiles in multistage carcinogenesis. *Proc Natl Acad Sci USA* **88:** 946–950, 1991.
15. Nagai F, Ushiyama K and Kano I, DNA cleavage by metabolites of butylated hydroxytoluene. *Arch Toxicol* **67:** 552–557, 1993.
16. Li Y and Trush MA, Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. *Cancer Res* **54:** 1895S–1898S, 1994.
17. Thompson CB, Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456–1462, 1995.
18. Bursch W, Oberhammer F and Schulte-Hermann R, Cell death by apoptosis and its protective role against disease. *Trends Pharm Sci* **13:** 245–251, 1992.
19. Marino AA and Mitchell JT, Lung damage in mice following intraperitoneal (i.p.) injection of butylated hydroxytoluene. *Proc Soc Exp Biol Med* **140:** 122–125, 1972.
20. Kharasch MS and Joshi BS, Reactions of hindered phenols. II. Base-catalyzed oxidations of hindered phenols. *J Org Chem* **22:** 1439–1443, 1957.
21. Coppinger GM and Campbell TW, Reaction between 2,6-di-*t*-butyl-*p*-cresol and bromine. *J Am Chem Soc* **75:** 734–736, 1953.
22. Chumakov P, EMBL Data Library, accession number X54156, 1990.
23. Capon DJ, Chen EY, Levinson AD, Seeburg PH and Goeddel DV, Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homolog. *Nature* **302:** 33–37, 1983.

24. Yamamoto K and Kawanishi S, Site-specific DNA damage induced by hydrazine in the presence of manganese and copper ions: the role of hydroxyl radical and hydrogen atom. *J Biol Chem* **266:** 1509–1515, 1991.
25. Kawanishi S and Yamamoto K, Mechanism of site-specific DNA damage induced by methylhydrazines in the presence of copper(II) or manganese(III). *Biochemistry* **30:** 3069–3075, 1991.
26. Maxam AM and Gilbert W, Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* **65:** 499–560, 1980.
27. Ito K, Yamamoto K and Kawanishi S, Manganese-mediated oxidative damage of cellular and isolated DNA by isoniazid and related hydrazines: non-Fenton-type hydroxyl radical formation. *Biochemistry* **31:** 11606–11613, 1992.
28. Ubezio P and Civoli F, Flow cytometric detection of hydrogen peroxide production induced by doxorubicin in cancer cells. *Free Radic Biol Med* **16:** 509–516, 1994.
29. Boissy RE, Trinkle LS and Nordlund JJ, Separation of pigmented and albino melanocytes and the concomitant evaluation of endogenous peroxide content using flow cytometry. *Cytometry* **10:** 779–787, 1989.
30. Oikawa S and Kawanishi S, Site-specific DNA damage induced by NADH in the presence of copper(II): role of active oxygen species. *Biochemistry* **35:** 4584–4590, 1996.
31. Taffe BG, Zweier JL, Pannell LK and Kensler TW, Generation of reactive intermediates from the tumor promoter butylated hydroxytoluene hydroperoxide in isolated murine keratinocytes or by hematin. *Carcinogenesis* **10:** 1261–1268, 1989.
32. Wand MD and Thompson JA, Cytochrome P-450-catalyzed rearrangement of a peroxyquinol derived from butylated hydroxytoluene. Involvement of radical and cationic intermediates. *J Biol Chem* **261:** 14049–14056, 1986.
33. Inouye S, Site-specific cleavage of double-stranded DNA by hydroperoxide of linoleic acid. *FEBS Lett* **172:** 231–234, 1984.
34. Inoue S and Kawanishi S, Hydroxyl radical production and human DNA damage induced by ferric nitrilotriacetate and hydrogen peroxide. *Cancer Res* **47:** 6522–6527, 1987.
35. Celander DW and Cech TR, Iron(II)-EDTA catalyzed cleavage of RNA and DNA oligonucleotides: similar reactivity toward single- and double-stranded forms. *Biochemistry* **29:** 1355–1361, 1990.
36. Sugiyama H and Saito I, Theoretical studies of GG-specific photocleavage of DNA via electron transfer: significant lowering of ionization potential and 5′-localization of HOMO of stacked GG bases in B form DNA. *J Am Chem Soc* **118:** 7063–7068, 1996.
37. Guyton KZ, Thompson JA and Kensler TW, Role of quinone methide in the *in vitro* toxicity of the skin tumor promoter butylated hydroxytoluene hydroperoxide. *Chem Res Toxicol* **6:** 731–738, 1993.
38. Land EJ and Swallow AJ, One-electron reactions in biochemical systems as studied by pulse radiolysis. IV. Oxidation of dihydroNAD. *Biochim Biophys Acta* **234:** 34–42, 1971.
39. Testa B, The metabolism of drugs and other xenobiotics: Biochemistry of redox reactions. In: The Metabolism of Drugs and Other Xenobiotics (Eds. Testa B and Caldwell J), pp 448–459. Academic Press, San Diego, 1995.
40. Kawanishi S, Oikawa S and Inoue S, Role of metal in oxidative DNA damage by non-mutagenic carcinogen. In: Genetic Response to Metal (Ed. Sarker B), pp 131–151. Marcel Dekker, Inc., New York, 1995.
41. Naito S, Ono Y, Somiya I, Inoue S, Ito K, Yamamoto K and Kawanishi S, Role of active oxygen species in DNA damage by pentachlorophenol metabolites. *Mutat Res* **310:** 79–88, 1994.
42. Hansson M, Asea A, Ersson U, Hermodsson S and Hellstrand K, Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. *J Immunol* **156:** 42–47, 1996.
43. Ueda N and Shah SV, Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J Clinical Invest* **90:** 2593–2597, 1992.
44. Dijkwel PA and Wenink PW, Structural integrity of the nuclear matrix: differential effects of thiol agents and metal chelators. *J Cell Sci* **84:** 53–67, 1986.
45. Burkitt MJ, Copper-DNA adducts. *Methods Enzymol* **234:** 66–79, 1994.
46. Chiu S, Xue L, Friedman LR and Oleinick NL, Differential dependence on chromatin structure for copper and iron ion induction of DNA double-stand breaks. *Biochemistry* **34:** 2653–2661, 1995.
47. Guyton KZ and Kensler TW, Oxidative mechanisms in carcinogenesis. *Br Med Bull* **49:** 523–544, 1993.
48. Oikawa S, Kurasaki M, Kojima Y and Kawanishi S, Oxidative and nonoxidative mechanisms of site-specific DNA cleavage induced by copper-containing metallothioneins. *Biochemistry* **34:** 8763–8770, 1995.
49. Hiraku Y, Inoue S, Oikawa S, Yamamoto K, Tada S, Nishino K and Kawanishi S, Metal-mediated oxidative damage to cellular and isolated DNA by certain tryptophan metabolites. *Carcinogenesis* **16:** 349–356, 1995.
50. Strasser A, Harris AW, Jacks T and Cory S, DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by *Bcl-2*. *Cell* **79:** 329–339, 1994.