

Molecular Mechanisms of Butylated Hydroxyanisole-Induced Toxicity: Induction of Apoptosis through Direct Release of Cytochrome *c*

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ABSTRACT

Butylated hydroxyanisole (BHA), a commonly used food preservative, is reported to have anticarcinogenic properties in some animal models. However, the use of BHA as a chemopreventive agent against cancer in human has been challenged by the observation that BHA may exert toxic effect in some tissues of animals. Therefore, it is of great significance to understand the mechanism of BHA-induced toxicity. Here, we report that BHA induces apoptosis in freshly isolated rat hepatocytes. Treatment of hepatocytes with BHA also induced loss of mitochondrial transmembrane potential ($\Delta\psi_m$), cytochrome *c*, and activation of caspase-3, -8, and -9 but not caspase-1. Pretreatment with cyclosporin A, an agent that stabilizes mitochondrial permeability transition pore, inhibited BHA-induced

loss of $\Delta\psi_m$, cytochrome *c* release, caspase activation, and apoptosis. Interestingly, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone failed to prevent these mitochondrial events, although it blocked caspase activation and apoptosis. Furthermore, BHA-induced apoptosis appeared to be independent of formation of reactive intermediates, as evidenced by the lack of effects of antioxidants *N*-acetyl-L-cysteine and ascorbic acid. Indeed, direct incubation of BHA with isolated mitochondria triggered cytochrome *c* release. Thus, these results indicate that the cytotoxicity of BHA is due to the induction of apoptosis that is mediated by the direct release of cytochrome *c* and the subsequent activation of caspases.

Apoptosis is a genetically regulated cell death that plays an essential role in the development and maintenance of homeostasis and protection against microbial or virus infection (Ellis et al., 1991); however, inappropriate activation of apoptotic program in the normal cells by physiological or environmental stimuli may cause toxic effects and serious diseases (Thompson, 1995). Apoptotic cells are characterized by plasma membrane blebbing, cell shrinkage, chromosomal DNA digestion, chromatin condensation, and formation of membrane-bound apoptotic bodies (Kerr et al., 1972). In the past few years, our understanding of molecular events involved in apoptosis has greatly progressed. A growing body of evidence indicates that mitochondria may serve a common mediator in the apoptosis induced by a variety of apoptotic stimuli (Susin et al., 1997; Yang et al., 1997; Green and Reed, 1998). One of the established pathways through which mitochondria regulate apoptosis is the release of cytochrome *c* from their intermembrane space. Once released to the cy-

tosol, cytochrome *c* binds to Apaf-1, a human homolog of *Caenorhabditis elegans* CED-4 protein, promoting further association with pro-caspase-9 (Zou et al., 1997; Saleh et al., 1999). Formation of such a protein complex results in proteolytic activation of caspase-9 that, in turn, activates downstream caspases, such as caspase-3, leading to apoptosis (Slee et al., 1999).

Butylated hydroxyanisole (BHA) is a synthetic phenolic antioxidant that has been primarily used as a food preservative due to its chain-breaking action in the lipid peroxidation (Kahl, 1984). In addition to the inhibition of lipid peroxidation, a number of studies in animals reveal that this compound exhibits a wide range of biological activities. Dietary administration of BHA protects animals against radiation and acute toxicity of radiation and various xenobiotics and prevents chemical-induced tumorigenesis in some animal tissues (Wattenberg, 1973; Kahl, 1984). These protective actions of BHA have been presumably attributed to its ability to induce phase II detoxifying enzymes such as epoxide hydrolases, glutathione *S*-transferases, UDP-glucuronosyltransferases, and quinone reductases (Benson et al., 1980;

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ABBREVIATIONS: BHA, butylated hydroxyanisole; tBHQ, *tert*-butylhydroquinone; CsA, cyclosporin A; NAC, *N*-acetyl-L-cysteine; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; DAPI, 4,6-diamidino-2-phenylindole; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; $\Delta\psi_m$, mitochondrial transmembrane potential; MPT, mitochondrial permeability transition; AMC, amino-4-methyl-coumarin.

Moldéus et al., 1982), and the ability to inhibit cytochrome p450 and monooxygenases that activate carcinogens (Cumings and Prough, 1983). In contrast to its beneficial effects, BHA is also found to be toxic and even carcinogenic in some animal models. For example, oral administration of high doses of BHA has been shown to cause cytotoxicity and to enhance the development of preneoplastic and neoplastic lesions in mouse forestomach and urinary bladder (Nera et al., 1988). Chronic exposure to high doses of BHA in diet has also been shown to induce papilloma and carcinoma formation in the forestomachs and esophagus of rats, mice, hamsters, and pigs (Ito et al., 1983; Wurzen and Olsen, 1986). Consistent with these results, we have previously shown that BHA and its metabolite, *tert*-butylhydroquinone, exerted a dose-dependent toxic effect in human hepatoma HepG2 and cervical squamous carcinoma HeLa cells (Yu et al., 1997).

Although the cytotoxicity of BHA has been well documented, the mechanism by which BHA induces cell death is not clear. Because BHA is not an electrophile, metabolic activation of BHA has been implicated in its toxic property (Kahl et al., 1989; Schilderman et al., 1993). However, the studies with isolated rat hepatocytes suggest that cell death induced by BHA does not require formation of reactive metabolites, but, rather, involves the release of calcium from mitochondria and the inhibition of respiration (Thompson and Moldéus, 1988; Nakagawa et al., 1994). Thus, in this study, we investigated the mechanisms of BHA-induced cytotoxicity in freshly isolated hepatocytes.

Materials and Methods

Reagents. Mouse anti-cytochrome *c* monoclonal antibody was purchased from Pharmingen (San Diego, CA). Fluorogenic peptide substrates of caspase-1 (Ac-YVAD-MCA) and caspase-3 (Ac-DEVD-MCA) were purchased from Peptides International (Louisville, KY). Fluorogenic peptide substrate of caspase-8 (Ac-IETD-MCA) was purchased from Upstate Biotechnology Institute (Lake Placid, NY). Fluorogenic peptide substrate of caspase-9 (Ac-LEHD-MCA) and a general caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), were purchased from Calbiochem (La Jolla, CA). Amino-4-methyl-coumarin (AMC) was purchased from Peninsula Laboratories (Belmont, CA). 3,3'-Dihexyloxacarbocyanine iodide [DiOC₆(3)] was purchased from Molecular Probes (Eugene, OR). BHA, *tert*-butylhydroquinone (tBHQ), cyclosporin A (CsA), ascorbic acid, *N*-acetyl-L-cysteine (NAC), diamidino-2-phenylindole (DAPI), and collagenase were purchased from Sigma (St. Louis, MO).

Isolation and Culture of Rat Hepatocytes. Male Fischer-344 rats (200–250 g) were fed ad libitum and fasted overnight before sacrifice. Hepatocytes were isolated by collagenase perfusion. The hepatocytes (approximately 90% viability as determined by trypan blue exclusion) were plated into 6-well Petri dishes at a density of 2×10^5 cells/well. For cytotoxicity assays, cells were cultured in 96-well plates. After incubation for 3 h at 37°C in an atmosphere of 5% CO₂ and high humidity, the cultures were washed with HEPES buffer (pH 7.4) to remove unattached dead cells. The cultures were then incubated for an additional 12 h in complete Williams E medium supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 0.1 U insulin, 1 µg/ml glucagon, and 100 U/ml penicillin/streptomycin, before treatments.

Cytotoxicity Assay. Hepatocytes were plated at a density of 10^4 -cells/100 µl/well into 96-well plates. After overnight culture, cells were treated with a series of concentrations of BHA (dissolved in Me₂SO, the final concentrations of which in culture medium was less than 0.1%) for various time periods. Cell viability was assayed with CellTiter 96 nonradioactive cell proliferation assay kit (Promega,

Madison, WI). Briefly, 20 µl of combined solution of a tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt], and an electron coupling reagent phenazine methosulfate was added to each well. After incubation for 1 h at 37°C in a humidified 5% CO₂ atmosphere, the absorbance at 490 nm was measured on an enzyme-linked immunosorbent assay plate reader.

DNA Fragmentation Assays. After treatment with BHA, hepatocytes (approximately 10^5 cells) were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 12,500g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed by agarose gel electrophoresis as described previously (Yu et al., 1998).

Nuclear Staining Assays. After treatments, hepatocytes were scraped off the plates and collected by centrifugation at 1,000g for 5 min. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining as described previously (Yu et al., 1998). Briefly, cells were washed once with ice-cold phosphate-buffered saline and fixed methanol:acetic acid (3:1) solution for 30 min. Fixed cells were placed on slides and stained 1 µg/ml DAPI for 15 min. Nuclear morphology of cells was examined by a fluorescence microscopy.

Caspase Activity Assay. After treatments, hepatocytes were washed twice with ice-cold PBS and lysed in a hypotonic buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM MgCl₂, 15 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 150 µg/ml digitonin. Cell lysates was homogenized by passing through a 23-gauge needle three times. Homogenates were kept on ice for 30 min and then centrifuged at 12,500g for 20 min at 4°C. The supernatants were transferred into the new tubes, and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA). The enzymatic activities of caspase-1, -3, -8, and -9 were assayed with their respective fluorogenic substrates as described previously (Yu et al., 1998).

Measurement of Mitochondrial Membrane Potential. After BHA treatment, hepatocytes were incubated with 40 nM DiOC₆(3) for 15 min at 37°C. The hepatocytes were then washed with ice-cold PBS and scraped off the plates in 1 ml of PBS. Cells were collected by centrifugation at 500g for 5 min and resuspended in 500 µl of PBS containing 2% fetal bovine serum and 40 nM DiOC₆(3). Fluorescence intensities of DiOC₆(3) were analyzed on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) with excitation and emission settings of 484 and 500 nm, respectively. Propidium iodide was added to the sample to gate out dead cells before data collection.

Preparation of Mitochondria from Rat Liver. Approximately 0.2 g of rat liver pieces was minced on ice and homogenized with a glass Dounce homogenizer in 1 ml of buffer A containing 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, and 1 mM EDTA. Homogenates were kept on ice for 20 min and centrifuged at 5,000g for 10 min at 4°C. The supernatants were recentrifuged at 20,000g for 30 min at 4°C. Mitochondrial pellets were resuspended in 500 µl of buffer B (200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 0.1% bovine serum albumin, and 10 mM HEPES-KOH, pH 7.4). Mitochondria were further purified by sucrose gradient (upper layer: 1.0 M sucrose in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5; Lower layer: 1.5 M sucrose in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5) (Spector, 1998). Interphase containing mitochondria was collected, washed once with buffer B, and resuspended in the same buffer. Protein concentration of mitochondrial fraction was determined by the Bradford method (Bio-Rad).

Preparation of S-100 Cytosolic Extracts and Western Blotting Analysis of Cytochrome *c* Release. Hepatocytes were washed twice with ice-cold PBS and scraped off the plates. Cells were collected by centrifugation at 500g for 5 min. The cell pellet was resuspended in 500 µl of extraction buffer containing 210 mM mannitol, 70 mM sucrose, 20 mM HEPES-KOH, pH 7.4, 50 mM KCl, 5

mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete Cocktail; Roche Molecular Biochemicals, Indianapolis, IN). After incubation on ice for 30 min, cells were homogenized with a glass Dounce and a B pestle. Homogenates were centrifuged at 12,000g for 5 min at 4°C. Supernatants were collected and further centrifuged at 100,000g for 30 min at 4°C to yield cytosol. Twenty-five micrograms of total protein, as determined by the Bradford method (Bio-Rad), was resolved on 15% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane using a semidry transfer system (Fisher). Membrane was blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCl, pH 7.4, 8 g/liter NaCl) for 1 h at room temperature, followed by incubation with 1 µg/ml primary monoclonal anti-cytochrome *c* antibody in TBS containing 3% nonfat milk for 1 h at room temperature. Membrane was washed three times with TBS and blotted with secondary antibody conjugated with horseradish peroxidase (1:10,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. The protein was visualized using an ECL system (Amersham) after washing three times in TBS.

Statistics. Data were expressed as means ± S.E. Statistical significance was determined by ANOVA. Comparison between the treatments and the control was conducted by using Duncan's multiple range test. A *P* value of less than .05 was considered significant.

Results

Treatment with BHA Causes Dose-Dependent Cytotoxicities in Rat Hepatocytes. Previous studies have shown that the biological activities of BHA, such as beneficial versus adverse effects, depends on the dosage of BHA used in animal models. Accordingly, we examined the effects of different concentrations of BHA on cell viability in the primary-cultured rat hepatocytes. As shown in Fig. 1, BHA at 500 µM caused a dramatic toxic response. Approximately 65% of cells lost their viability within 8 h of BHA treatment. Increasing BHA concentration to 1 mM caused a more vigorous response, and over 90% cells lost the viability after incubation for the same time period. In contrast, no apparent toxic effect was observed for BHA at 125 µM, even up to 24 h of treat-

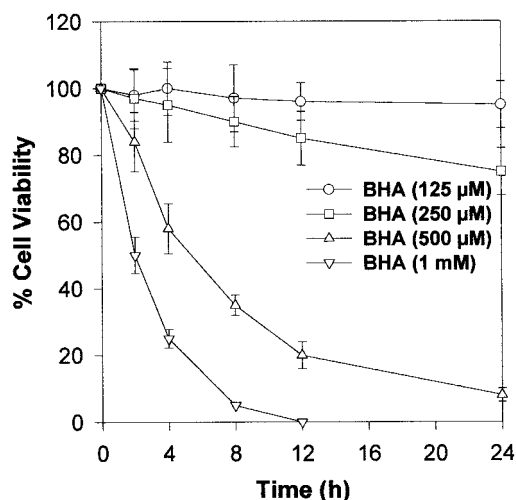


Fig. 1. Effect of BHA on rat hepatocyte viability. Rat hepatocytes were treated with different concentrations of BHA for various time periods. Cell viability then was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay as described. The percentage of cell viability was calculated as a ratio of A_{490 nm} of treated cells and control cells (treated with 0.1% Me₂SO, a solvent). Data presented are means ± S.E. (*n* = 6).

ment. Furthermore, BHA at 250 µM only slightly reduced hepatocyte viability. Similar results were obtained when cells were assayed by trypan blue exclusion (data not shown), indicating that the loss of viability was due to the cell death induced by BHA.

BHA Induces Apoptosis in Rat Hepatocytes. Cell death can be distinguished, generally, as necrotic and apoptotic. A necrotic cell death is associated with cell swelling and disruption of cell membrane, without severe damage to the nuclei or breakdown of DNA. Apoptotic cell death is characterized by apoptotic morphologies such as membrane blebbing, internucleosomal degradation of DNA, and chromatin condensation. To characterize cell death induced by BHA, we examined the nuclear morphology of dying cells. As determined by nuclear staining with DAPI, treatment with BHA (500 µM for 8 h) induced extensive condensation and fragmentation of nuclei (Fig. 2A), whereas the control cells (treated with 0.1% Me₂SO) showed no morphological change. Further analysis of dying cells for DNA laddering revealed that BHA also induced digestion of genomic DNA (Fig. 2B). Interestingly, BHA at 1 mM resulted in different DNA fragmentation. This is probably due to the reduced recovery of all fragmented DNA resulted from the acute toxicity and rapid damage of cell membrane by the high concentrations of BHA.

Caspases Mediate BHA-Induced Apoptosis. Recent studies have identified caspases as important mediators of apoptosis induced by various apoptotic stimuli. To determine the roles of caspases in BHA-induced apoptosis, we measured the activities of several caspases in BHA-treated rat hepatocytes. As shown in Fig. 3A, BHA (500 µM) strongly stimulated caspase-3-like protease activities. The activation of caspase-3 was time-dependent, with a maximum of 15-fold induction being observed by 2 h of BHA treatment. Activities of caspase-8 and -9 were also induced by BHA but showed different time courses. A significant increase in caspase-9 activity (approximately 2-fold over control) was seen 30 min post-treatment with BHA, whereas on induction of caspase-3, activity was undetectable at this time point, suggesting that

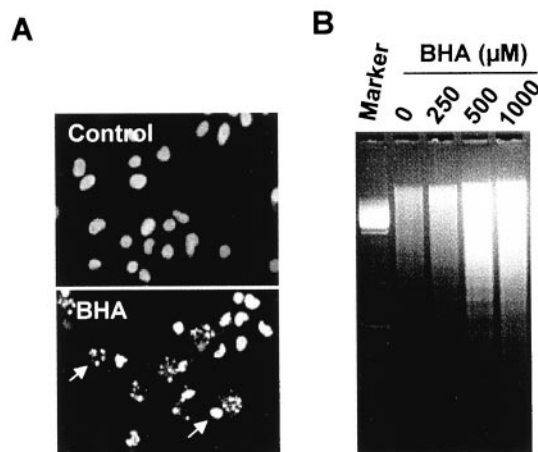


Fig. 2. Induction of apoptosis by BHA in rat hepatocytes. A, nuclear morphology. Rat hepatocytes were treated with BHA (500 µM) for 8 h or with 0.1% Me₂SO as control. Cells were harvested and fixed in methanol-acetic acid (3:1) solution. Nuclear morphology of cells was visualized by DAPI staining. Arrows indicate condensed or fragmented nuclei. B, genome digestion. Hepatocytes were treated with the indicated concentrations of BHA for 8 h. Cellular DNA was extracted and analyzed by agarose gel electrophoresis. Data shown are representatives of three separate experiments.

caspase-9 activation preceded caspase-3 activation. However, as compared with caspase-3 activation, caspase-8 activation was delayed. In addition, no significant change in caspase-1 activity was observed throughout the time course study.

To address the significance of caspase activation in BHA-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone). Blockade of caspase activities by pretreatment of hepatocytes with z-VAD-fmk attenuated BHA-induced genomic DNA digestion in a dose-dependent manner (Fig. 3B).

BHA Triggers Mitochondrial Permeability Transition, Which Is Upstream of Caspases. It is known that

caspase cascades can be initiated by at least two pathways. One is death receptor-mediated caspase-8 pathway (Ashkenazi and Dixit, 1998). The other is mitochondria-mediated caspase-9 pathway (Green and Reed, 1998). Because the activation of caspase-9 by BHA preceded the activation of other caspases (Fig. 3A), we therefore examined the involvement of mitochondria. Rat hepatocytes were treated with BHA (500 μ M) and stained with DiOC₆(3) to assess mitochondrial transmembrane potential ($\Delta\psi_m$). Hepatocytes showed a substantial loss of $\Delta\psi_m$ within 30 min of BHA treatment (Fig. 4A). BHA-induced loss of $\Delta\psi_m$ was attenuated by pretreatment with CsA, an inhibitor of mitochondrial permeability transition (MPT) pore opening. CsA also inhibited activation of caspase-3, -8 and -9 (Fig. 4B). Interestingly,

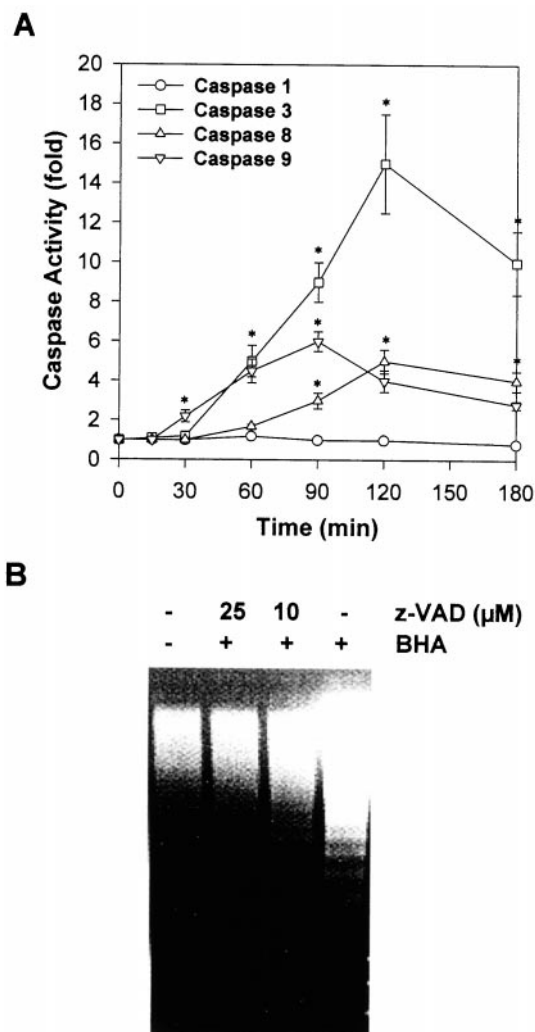


Fig. 3. Role of caspases in BHA-induced apoptosis. A, induction of caspase activity. Rat hepatocytes were treated with BHA (500 μ M) for different time periods and harvested in lysis buffer. Enzymatic activities of caspase-1, -3, -8, and -9 were determined by incubation of 10 μ g of total protein with 200 μ M fluorogenic substrates, Ac-YVAD-MCA, Ac-DEVD-MCA, Ac-IETD-MCA, and Ac-LEHD-MCA, in a 50- μ l assay buffer for 2 h at 37°C, respectively. The release of AMC was monitored spectrofluorometrically (excitation = 360 nm; emission = 460 nm). Data presented are means \pm S.E. (n = 4). Superscript asterisk (*) indicates a significant difference at P < .05 as compared with control. B, inhibition of BHA-induced genome digestion by z-VAD-fmk. Rat hepatocytes were treated with different concentrations of z-VAD-fmk for 1 h before challenge with 500 μ M BHA for 8 h. Cellular DNA was extracted and analyzed by agarose gel electrophoresis. Data shown are a sample of two separate experiments.

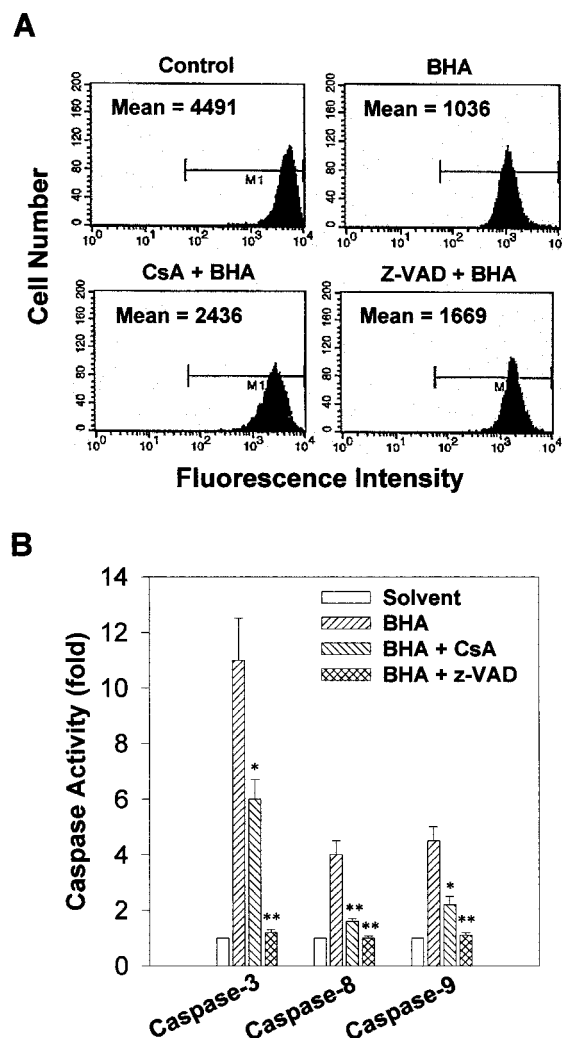


Fig. 4. Reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) by BHA is upstream of caspases. A, effects of CsA and z-VAD-fmk on BHA-induced reduction of $\Delta\psi_m$. Rat hepatocytes were incubated with CsA (0.5 μ g/ml), z-VAD-fmk (25 μ M), or solvent (0.1% Me₂SO) for 1 h before treatment with BHA (500 μ M) for 30 min. DiOC₆(3) was added to the culture medium during the last 15 min of treatment at a final concentration of 40 nM. The fluorescence intensity of DiOC₆(3) was analyzed by a flow cytometry. B, effects of CsA and z-VAD-fmk on BHA-induced caspase activation. Rat hepatocytes were incubated with CsA, z-VAD-fmk, or solvent, as described in A, before treatment with BHA (500 μ M) for 2 h. Caspase activity was determined as described under *Materials and Methods*. Data shown are means \pm S.E. (n = 3). Asterisks (*) and (**) indicate significant difference at P < .05 and < 0.01, respectively, compared with the treatment with BHA alone.

although z-VAD-fmk abolished BHA-induced caspase activities (Fig. 4B), it had little effect on BHA-induced loss of $\Delta\psi_m$ (Fig. 4A). These results suggest that BHA-induced mitochondrial events are upstream of the caspases.

BHA Induces Cytochrome *c* Release in Rat Hepatocytes. Permeability transition pore opening has been shown to cause a release of proapoptotic proteins from the intermembrane space of mitochondria into cytosol. Cytochrome *c* that binds to Apaf-1 to initiate caspase cascades is among those released proteins (Slee et al., 1999). To examine the release of cytochrome *c* in BHA-treated hepatocytes, we conducted Western blotting analysis with the cytosolic fractions obtained by ultracentrifugation. BHA induced a time-dependent increase of cytochrome *c* in the cytosol (Fig. 5A), indicative of a release of cytochrome *c* from mitochondria. Pretreatment with CsA that prevented the loss of $\Delta\psi_m$ inhibited BHA-induced cytochrome *c* release (Fig. 5B). However, z-VAD-fmk had no effect on this event, supporting the notion that activation of caspases is downstream of mitochondria.

Induction of Apoptosis by BHA Appears to Be Independent of Formation of Reactive Metabolites. In mammalian cells, especially in hepatocytes, BHA is metabolized by several enzyme systems such as cytochrome P450s or peroxidases. One of the major metabolites of BHA, as shown in rats and humans, is a demethylated product, tBHQ, which can further generate reactive oxygen species through redox cycling (Astill et al., 1962). To examine the role of tBHQ and the reactive intermediates in BHA-induced apoptosis, we used the antioxidants, NAC and ascorbic acid, which have been shown previously to block the activation of extracellular signal-regulated protein kinase by BHA and tBHQ (Yu et al. 1997). Like BHA, tBHQ (500 μ M) induced apoptotic cell death in freshly isolated rat hepatocytes (Fig. 6). Pretreatment with NAC or ascorbic acid significantly inhibited tBHQ-induced apoptosis, but had little effect on BHA-induced apoptosis. This result suggests that BHA-induced apoptosis is not mediated by tBHQ or its reactive intermedi-

ates, although tBHQ is able to induce apoptosis. In addition, z-VAD-fmk completely blocked the apoptosis induced by BHA and tBHQ. CsA also inhibited the induction of apoptosis by both agents, although to a lesser extent compared with z-VAD-fmk.

BHA Induces Cytochrome *c* Release from Isolated Mitochondria. Because BHA-induced MPT and cytochrome *c* release is upstream of caspase activation, we next examined whether BHA had a direct effect on mitochondria. To this end, we incubated BHA with isolated rat mitochondria. Release of cytochrome *c* was analyzed by Western blotting. As shown in Fig. 7A, BHA induced a time-dependent release of cytochrome *c* from mitochondria, which became evident within 10 min of incubation. The induced cytochrome *c* release was inhibited by the addition of CsA but not z-VAD-fmk or NAC (Fig. 7B), consistent with the results obtained in the hepatocytes. These data substantiate a critical role of mitochondria in BHA-induced apoptosis.

Discussion

Our results show that BHA induces concentration-dependent apoptosis in freshly isolated rat hepatocytes. At the apoptosis-inducing concentrations, BHA also stimulates proteolytic activities of caspase-3, -8, and -9, and triggers MPT and release of cytochrome *c*. Inhibition of caspase activation by z-VAD-fmk or cytochrome *c* release by CsA attenuated BHA-induced apoptosis. Thus, the cytotoxicity of BHA in hepatocytes is due, at least in part, to the induction of apoptosis, which involves a cytochrome *c* and caspase-dependent mechanism.

A growing number of studies (Alnemri, 1997; Ashkenazi and Dixit, 1998; Green and Reed, 1998; Thornberry and Lazebnik, 1998) indicate that apoptotic signals either initiated at the cell membrane or in the cytosol converge on caspase cascades that are responsible for the biochemical and morphological changes in apoptotic cells. For example, in the death receptor-mediated apoptosis, engagement of death li-

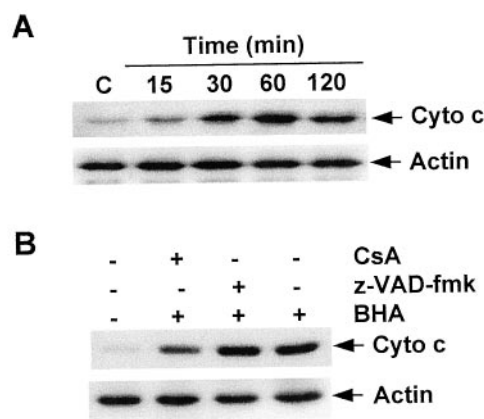


Fig. 5. Release of cytochrome *c* in BHA-treated hepatocytes. A, time-dependent release of cytochrome *c*. Rat hepatocytes were incubated with BHA (500 μ M) for different time periods. Cytosolic extracts were prepared as described under *Material and Methods*. Twenty-five micrograms of cytosolic protein was resolved on 15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blotted with anti-cytochrome *c* antibody, or with anti-actin antibody to serve as control for the loading of protein level. B, effects of CsA and z-VAD-fmk on BHA-induced cytochrome *c*. Hepatocytes were incubated with CsA (0.5 μ g/ml), or z-VAD-fmk (25 μ M), or solvent (0.1% Me_2SO) for 1 h before challenge with BHA (500 μ M) for 1 h. Cytosolic extracts were prepared and analyzed as described. Experiments were repeated three times.

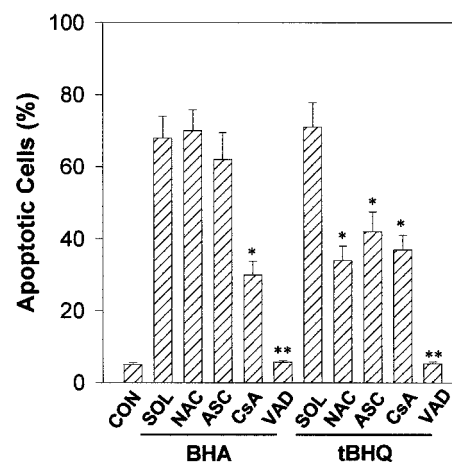


Fig. 6. Effects of antioxidants on BHA- and tBHQ-induced apoptosis. Rat hepatocytes were pretreated with NAC (15 mM), ascorbic acid (ASC, 100 μ M), CsA (0.5 μ g/ml), z-VAD-fmk (25 μ M), or solvent (SOL, 0.1% Me_2SO) for 1 h, and then treated with BHA (500 μ M) or tBHQ (500 μ M) for 8 h or left untreated as control (CON). The number of cells showing apoptotic morphology was determined by DAPI staining. Data presented are means \pm S.E. ($n = 3$). Asterisks (*) and (**) indicate significant difference at $P < .05$ and < 0.01 , respectively, compared with treatment with BHA or tBHQ alone.

gands such as FasL/Apo1 and tumor necrosis factor triggers oligmerization of death receptors, resulting in formation of a protein complex known as the DISC (death-inducing signaling complex), which consists of death receptors, adapter proteins such as TRADD or FADD, and pro-caspase-8. Once pro-caspase-8 is recruited to this complex, it is processed to the active form, presumably through the autocatalysis, and activates the downstream caspases such as caspase-3, leading to apoptosis (Srinivasula et al., 1996). In addition, activation of caspase-8 may cause cleavage of Bid that subsequently translocates to mitochondria and triggers cytochrome *c* release (Luo et al., 1998). Therefore, activation of mitochondria and release of cytochrome *c* in death ligand-induced apoptosis is secondary to caspase activation. However, in chemical- or radiation-induced apoptosis, cytochrome *c* release is not affected by caspase inhibitors (Green and Reed, 1998). Instead, the released cytochrome *c* stimulates the proteolytic activity of caspase-9 by association with Apaf-1, a human homolog of *C. elegans* Ced-4 protein, and further activates downstream caspases, such as caspase-3, causing apoptotic cell death (Slee et al., 1999). In this study, BHA-induced cytochrome *c* release preceded the activation of caspases and was insensitive to the caspase inhibitor, z-VAD-fmk. Furthermore, prevention of MPT and cytochrome *c* release by pretreatment with CsA inhibited BHA-induced caspase activation and apoptosis. Thus, the activation of mitochondria and release of cytochrome *c* is an early event in BHA-induced apoptosis and may be responsible for the initiation of apoptotic signals.

Mitochondria have been previously implicated in the BHA-induced cytotoxicity. In isolated rat mitochondria, BHA disrupts the membrane potential, inhibits respiratory control, induces calcium release, and causes mitochondria swelling (Thompson and Moldéus, 1988; Nakagawa et al., 1994). Our demonstration that BHA induces cytochrome *c* release provides further evidence for the role of mitochondria in BHA-induced cytotoxicity. However, the question remains as to how BHA triggers MPT and induces cytochrome *c* release. MPT is known to be induced by a variety of stimuli such as UV irradiation, chemical stresses, and oxidants (Susin et al.,

1997; Yang et al., 1997; Jiang et al., 1999). Although the precise mechanisms responsible for the induction of MPT by these stimuli are not clear, generation of oxidative stress is believed to be a common pathway (Costantini et al., 1996). In many types of cells, especially in hepatocytes, BHA is metabolized by cytochrome p450s or monooxygenase to tBHQ that, in turn, is converted to *tert*-butylquinone either through autoxidation or by the enzymes (Astill et al., 1962; Kahl et al., 1989). Formation of *tert*-butylquinone initiates redox-cycling, resulting in the production of reactive oxygen species (Kahl et al., 1989). Alternatively, BHA can be metabolized by peroxidases such as horseradish peroxidase and prostaglandin H synthase or cyclooxygenase to phenoxyl free radicals that are capable of binding to many macromolecules (Thompson et al., 1989; Schilderman et al., 1993). However, as shown in this study, scavenging the reactive intermediates by antioxidants NAC or ascorbic acid, which inhibited apoptosis induced by tBHQ, did not affect BHA-induced apoptosis, suggesting that BHA by itself may induce apoptosis. Indeed, direct incubation of BHA with isolated mitochondria caused cytochrome *c* release. It is therefore conceivable that BHA may directly act on mitochondria to initiate apoptotic signals. Earlier studies showed that BHA intercalated into the hydrophobic phase of membrane lipids (Eletr et al., 1974). Such interaction, on the one hand, prevents damage to lipid membranes by terminating free radical chain reactions (Kahl, 1984); on the other hand, this may interfere with membrane integrity and membrane-bound protein functions (Sokolove et al., 1986). Further studies should focus on the interaction of BHA with mitochondrial membrane and identify a mitochondrial target(s).

In summary, we demonstrate that the cytotoxicity of BHA in hepatocytes involves the induction of apoptosis. A possible mechanism for BHA-induced apoptosis is presented in Fig. 8. In the schematic, BHA interacts with mitochondria and triggers mitochondrial permeability transition, resulting in the

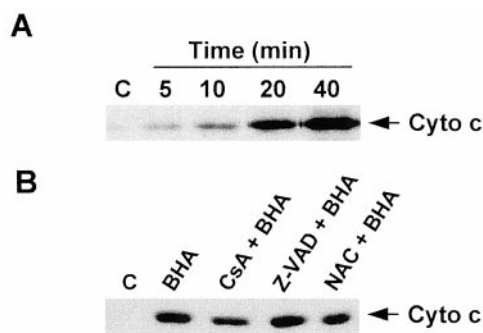


Fig. 7. Induction of cytochrome *c* release from isolated rat mitochondria by BHA. A, time course of cytochrome *c* release. Ten micrograms of isolated rat mitochondria was incubated with BHA (500 μ M) in a 25- μ l assay buffer for different time periods at 30°C. At the end of incubation, reaction mixture was centrifuged at 20,000g for 15 min at 4°C to pellet mitochondria. The resulting supernatant was analyzed by Western blotting with anti-cytochrome *c* antibody as described. B, effects of CsA, z-VAD-fmk, and NAC on BHA-induced cytochrome *c* release. Ten micrograms of isolated rat mitochondria was incubated with BHA (500 μ M) either alone or in the presence of CsA (0.5 μ g/ml), z-VAD-fmk (25 μ M), or NAC (5 mM) for 30 min at 30°C. The reaction mixture was centrifuged at 20,000g for 15 min at 4°C and analyzed as in A.

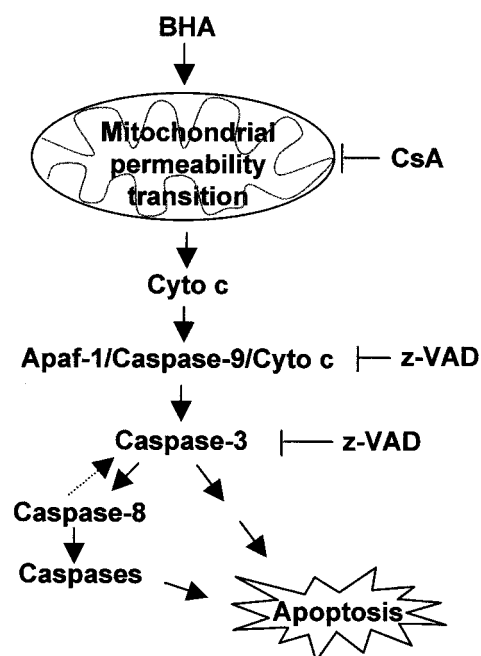


Fig. 8. A proposed mechanism for BHA-induced cytotoxicity in rat hepatocytes.

release of cytochrome *c*. Once in the cytosol, cytochrome *c* binds to Apaf-1, a human homolog of *C. elegans* Ced-4 protein, and promotes formation of a complex with procaspase-9, thereby stimulating the catalytic activity of caspase-9. Activated caspase-9, in turn, proteolytically activates downstream effectors, such as caspase-3, which further cleaves a number of cellular proteins, leading to apoptosis. In addition, caspase-3 may activate caspase-8, which subsequently activates other caspases, resulting in amplification of apoptotic signals. It has been noted that the biological activities of BHA are strongly dependent on the dosage used in the experiments (Kahl, 1984). Consistent with this notion, we showed that BHA at concentrations less than 200 μ M does not exert a significant toxic effect in hepatocytes (Fig. 1) but, instead, stimulates the induction of phase II detoxifying enzymes (Yu et al., 1999). Interestingly, a recent study (Kirlin et al., 1999) reports that BHA induces apoptosis in human colon carcinoma HT29 cells at much lower concentrations, which also induces phase II detoxifying enzymes, suggesting that cancerous cells may be more sensitive to BHA than normal cells. Therefore, understanding such differential responses of cancer cells and normal cells to BHA warrants further studies.

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