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The Antioxidant Butylated Hydroxytoluene Induces Apoptosis in Human U937 Cells: The Role of Hydrogen Peroxide and Altered Redox State

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Exposure of U937 cells to the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT), unlike exposure to other antioxidants such as *N,N'*-diphenyl-1,4-phenylenediamine, Trolox or α-tocopherol, promotes a time- and concentration-dependent induction of apoptosis. This response was prevented by the iron chelator *o*-phenanthroline and by the thiol reagent *N*-acetylcysteine but was increased remarkably in cells pre-exposed to the catalase inhibitor 3-amino-1,2,4-triazole or to L-buthionine-[S,R]-sulfoximine, a specific inhibitor of glutathione synthesis. Furthermore, the BHT-induced apoptotic response was markedly enhanced by cytochrome P450 inhibitors.

Taken together, the experimental results presented in this study indicate that BHT efficiently induces apoptosis in U937 cells and that this response is not caused by products of cytochrome P450 metabolism. Instead, apoptosis appeared to be causally linked to an altered cellular redox state in which hydrogen peroxide plays a pivotal role.

Keywords: 2,6-di-*tert*-butyl-4-methylphenol, antioxidant, DNA fragmentation, apoptosis, hydrogen peroxide

INTRODUCTION

The phenolic antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT), a commonly used human food additive, prevents the toxic effects promoted by various oxidizing agents in cultured mammalian cells.[1,2]

Several studies, however, have reported that BHT can be toxic in laboratory animals. For example, BHT was shown to induce destruction of type I alveolar and pulmonary endothelial cells in the mouse lung and liver necrosis in rats.[3–6] Cytochrome P4502B isoenzymes appear to be responsible for pulmonary bioactivation and toxicity of BHT.[7] In addition, BHT was reported to increase the incidence of tumors in mice and rats treated with various carcinogens[8–11] and to cause liver cancer in mice.[12] An oxidative metabolite of BHT, butylated hydroxytoluene hydroperoxide (BHTOOH), displays tumor-promoting

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activity in mouse skin.[13–15] BHTOOH is further metabolized to several reactive species, including free radical intermediates and non-radical, electrophilic quinone methides[16] such as 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone which most likely mediate the tumor promotion induced by BHTOOH.[17]

Thus, BHT on the one hand is cytoprotective for oxidatively injured cells, but on the other hand is metabolized to reactive intermediates and therefore promotes toxic responses as well. Although, these events have been described in detail in laboratory animals, little is known about the molecular mechanism by which BHT causes cell death. High concentrations of BHT were found to be toxic for cultured heart cells.[18] Thompson and Moldéus[19] reported that the lethal response of isolated rat hepatocytes to BHT is the consequence of effects on biomembranes and mitochondrial bioenergenetics.

The aim of this study was to investigate the mode of death induced by BHT in cultured U937 cells as well as the nature of the BHT-derived metabolites involved in the lethal response.

MATERIALS AND METHODS

Cell Culture and Treatments

U937 cells were cultured in suspension in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) (Sera-Lab Ltd., Crawley Down, England), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air–5% CO_2.

BHT was dissolved in 95% ethanol. At the treatment stage, the final ethanol concentration was never higher than 0.05%. Under these conditions, ethanol did not cause U937 cell death. Cells (5.0×10^5) were inoculated into 35 mm tissue culture dishes and exposed for 3 h to increasing concentrations of BHT. Controls received equal amounts of vehicle. After the treatments, the cells were washed with saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose), resuspended in pre-warmed culture medium, plated in 35 mm culture dishes and incubated at 37°C for different time intervals.

Cytotoxicity Assay

Cytotoxicity was determined using the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1 : 1 with 0.4% trypan blue and the cells were counted with a hemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells vs the total number of cells).

Catalase Activity

Cells were rinsed twice in saline A, resuspended in the same medium at a density of 1×10^6 cells/ml and finally sonicated three times on ice with a Heat Systems XL sonicator (Heat Systems Inc., Farmingdale, NY, USA) operating at 20 W for 15 s. The resulting homogenates were centrifuged for 5 min at 18,000 *g* at 4°C. Catalase activity was assayed spectrophotometrically in the supernatant by the method of Aebi.[20]

Non-protein Sulphydryl (NPSH) Assay

NPSH levels were determined using the 5,5′-dithiobis-(2-nitrobenzoic acid) method, as previously described.[21] The concentration of NPSH in the samples was determined by comparison with standard solutions of GSH. Protein contents were assayed as described by Lowry *et al.*[22] using bovine serum albumin as standard.

DNA Fragmentation Analysis by the Filter-binding Assay

Secondary DNA fragmentation was quantified using the filter-binding assay developed by

Bertrand *et al.*[23] with minor modifications.[24] DNA fragmentation was determined as the percent ratio of the [^{14}C]-labelled DNA eluted out of the filter vs the total DNA radioactivity in the sample.

DNA Fragmentation Analysis by Conventional Gel Electrophoresis

Genomic DNA was isolated from 3.5×10^6 U937 cells. Cells were sedimented in a 15 ml conical tube at 1800 rpm for 10 min (4°C) and the pellet was washed three times with 5 ml of prechilled phosphate-buffered saline (8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl) containing 5 mM ethylenediamine tetraacetic acid (EDTA), pH 8.3. Cells were lysed with 100 µl of buffer containing 10 mM Tris/HCl, 25 mM EDTA, 100 mM NaCl, 0.2% SDS and 0.5 mg/ml proteinase K; the cell suspension was then left in a 55°C water bath for 2 h. Agarose 2% (low melt agarose, Bio Rad, Richmond, CA, USA) was melted in Tris borate-EDTA (TBE) buffer (89 mM Tris base, 89 mM sodium borate, 2 mM Na_2EDTA [pH 8.3]) and 50 µl were added to the cell lysate. The mixture was quickly dispensed into a plug former on ice. The plugs were analyzed by electrophoresis using a 1.5% agarose gel in TBE for 4 h at 50 V. The gel was incubated with DNase-free RNase (20 µg/ml) at 37°C for 4 h before staining with ethidium bromide and then photographed under a UV transilluminator.

DNA Fragmentation Analysis by Programmable, Autonomously Controlled Electrodes (PACE) Electrophoresis

Cells were embedded and lysed into agarose plugs as previously described by Sestili *et al.*[25] PACE electrophoresis was carried out using a Bio Rad DRIII variable angle system. Briefly, the gels were cast using 1.0% w/v chromosomal grade agarose in 0.5x TBE buffer (composition of the 0.5x concentrated buffer: 44.5 mM Tris HCl, 44.5 mM boric acid, 1 mM Na_2EDTA [pH 8.3]) and run according to the three-block assay described by Sestili *et al.*[25] Gels were stained with ethidium bromide, viewed with a UV transilluminator and photographed.

Comet Assay

Apoptotic cells were scored using a method described by Fairbairn *et al.*[26] a slightly modified version of the comet assay. After treatments, U937 cells were resuspended at 2.0×10^4 cells/100 µl in 1.0% low-melting agarose in phosphate-buffered saline containing 5 mM EDTA and immediately pipetted onto agarose-coated slides. The slides were immersed in ice-cold lysing solution (1 M NaCl, 10 mM Tris, 0.1% sarkosyl, 0.03 M NaOH) for 60 min. The slides were then placed on an electrophoretic tray with 0.03 M NaOH as running buffer and electrophoresis was then performed at 0.7 V/cm for 20 min at 14°C.

Finally, slides were washed and stained with ethidium bromide. The DNA was visualized using a Bio Rad DVC 250 confocal laser microscope. Under these conditions apoptotic cells appeared as "tailed" cells, as compared to non-apoptotic, "untailed", cells. The number of apoptotic cells was calculated as the ratio of "tailed" (apoptotic) to "untailed" (non-apoptotic) cells. A minimum of 250 randomly selected cells per treatment condition was averaged.

Statistical Analysis

All data in tables and figures are expressed as mean ± SEM. For comparison between two groups the Student's upaired *t* test was used. When three or more groups were compared one-way ANOVA, followed by Dunnett's test for multiple comparisons, was used.

RESULTS

When U937 cells were exposed to 100, 150 or 200 µM BHT for 3 h and then incubated under

growth conditions, the number of viable cells (as measured by the trypan blue exclusion assay) was always lower than the control (Figure 1A). In particular, treatment with either 150 or 200 μM BHT led to a remarkable decline in the number of viable cells. Evidence of cell proliferation was observed only after 40 h of post-treatment incubation. These events were mirrored by the appearance of dead cells which was maximal after 20 h (Figure 1B). The relative number of dead cells was found to decrease after 40 h, as a result of both the lysis of the dead cells and the proliferation of the viable cells.

BHT, while not causing loss of viability (not shown) during the 3 h of exposure, produced significant morphological changes. Light microscopy (Figure 2) demonstrated that U937 cells (control in panel A) exposed for 3 h to 200 μM BHT (panel B) show extensive blebbing, a typical morphological change associated with apoptosis. Necrotic cells were not detected.

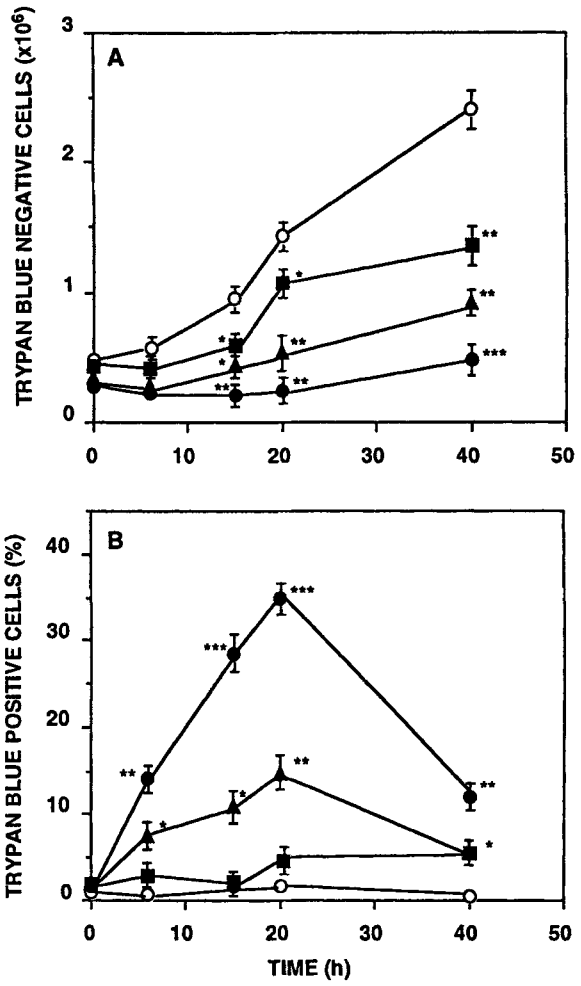

FIGURE 1 BHT-induced U937 cell death. Cells were treated for 3 h with 0 (open circles), 100 (closed squares), 150 (closed triangles) or 200 μM (closed circles) BHT and then post-incubated in complete drug-free medium. Viable (panel A) and unviable (panel B) cells were counted at different time intervals using the trypan blue exclusion assay. Results are the mean ± SEM calculated from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ compared with untreated cells by analysis of variance followed by Dunnett's test.

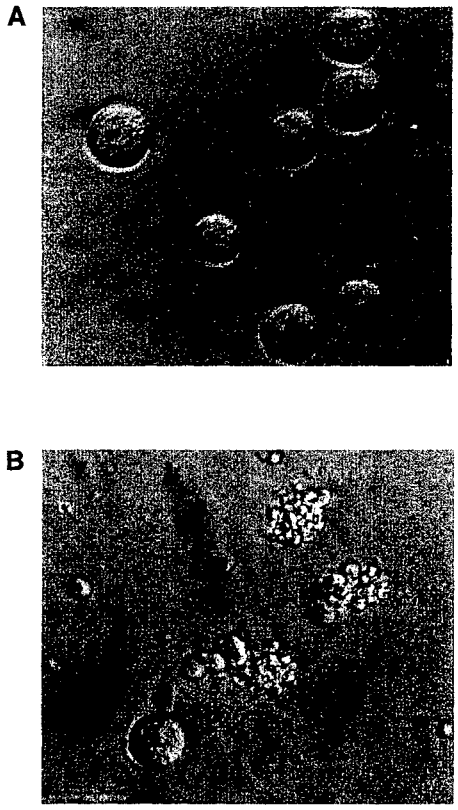

FIGURE 2 Morphological changes induced by BHT in U937 cells. Cells were exposed for 3 h to 0 (panel A) or 200 μM (panel B) BHT and then photographed using an Olympus IX-70 inverted microscope. The photomicrographs are representative of three experiments with similar outcomes.

Figure 3 shows that BHT also generates secondary DNA fragmentation, as measured by the filter binding assay. Treatment for 3 h with increasing concentrations of the antioxidant produced DNA fragmentation that was concentration-dependent over a range of 100–200 μM (panel A). BHT (200 μM) generated a detectable level of DNA fragmentation after only 1 h of exposure and then progressively increased for up to 3 h (panel B).

These results strongly suggest that BHT induces apoptosis in a time- and concentration-dependent fashion and are in general agreement with those obtained using a modification of the comet assay,[26] which allows the detection and quantitation of apoptotic cells (Figure 3C and D). The results obtained with conventional gel electrophoresis and PACE, indicating that treatment with 150 or 200 μM BHT promotes the formation of DNA laddering (Figure 4A) and ≤ 50 kb double-stranded DNA fragments (Figure 4B), are also consistent with this conclusion.

Thus, convincing experimental evidence demonstrates that BHT is toxic for cultured U937 cells and that the mechanism of BHT-induced cell death in this cell line is apoptosis. We observed that BHT induced apoptosis also in PC12 cells (not shown).

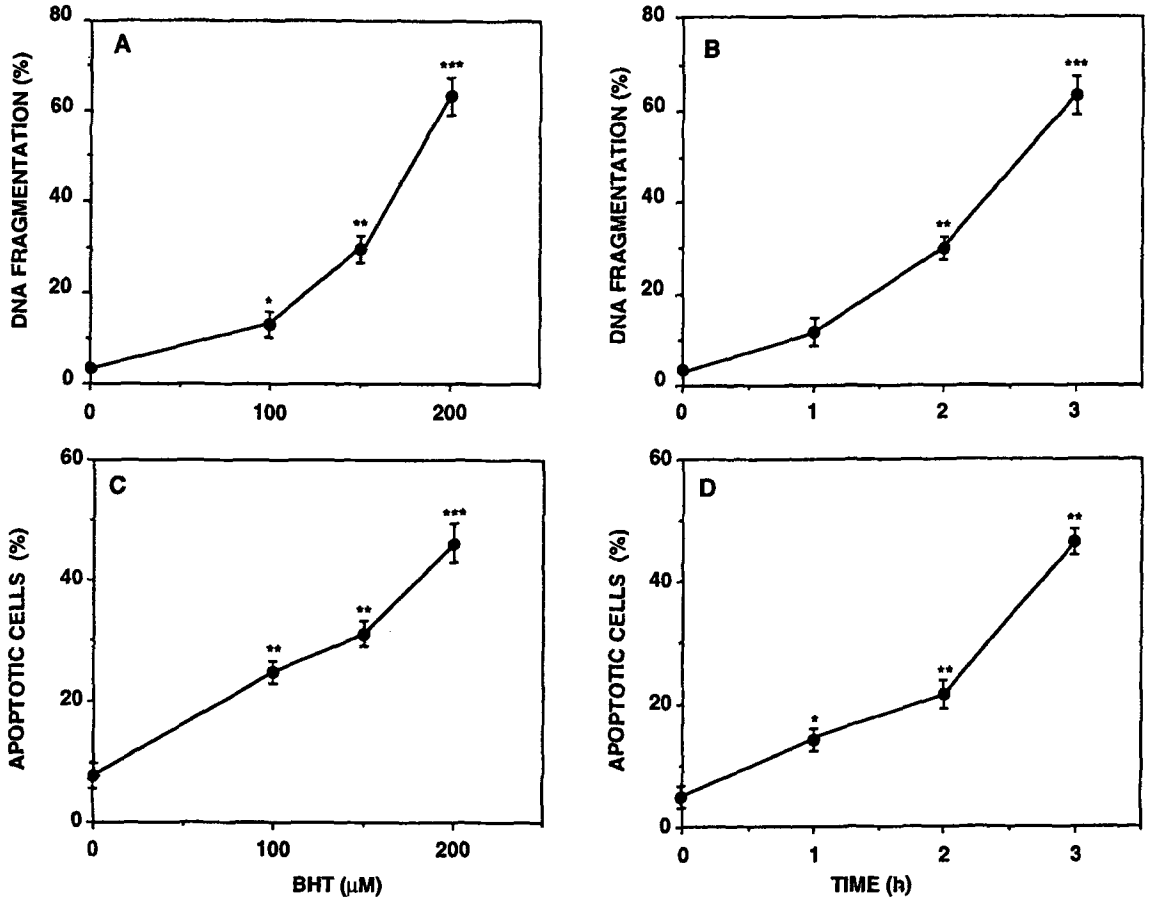

FIGURE 3 BHT-induced secondary DNA fragmentation and apoptotic cell death. Cells were treated with different concentrations of BHT for 3 h (panels A and C) or for different lengths of time with 200 μM BHT (panels B and D). The percentage of secondary DNA fragmentation (panels A and B) and of apoptotic cells (panels C and D) was estimated using the filter-binding and comet assays, respectively, as described in the Materials and Methods section. Results are the mean ± SEM calculated from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ vs untreated cells (unpaired t test).

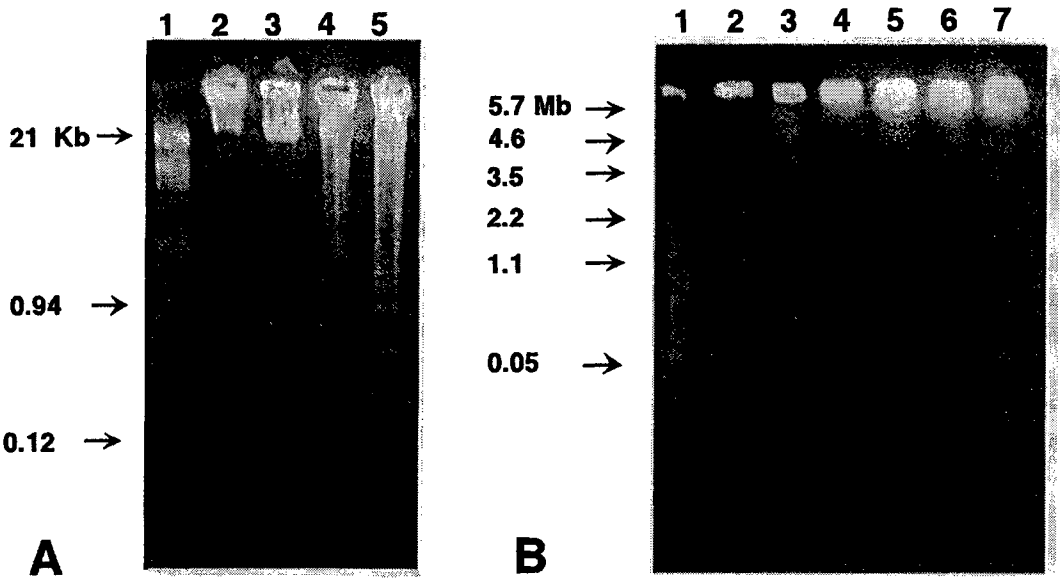

FIGURE 4 Conventional and PACE agarose gel electrophoretic analysis of the DNA from U937 cells treated with increasing concentrations of BHT. Cells were exposed for 3 h to 0 (lane 2, panel A and lane 4, panel B), 100 (lane 3, panel A and lane 5, panel B), 150 (lane 4, panel A and lane 6, panel B) or 200 μM BHT (lane 5, panel A and lane 7, panel B) and then the DNA was analyzed by conventional (panel A) and PACE (panel B) gel electrophoresis as described in the Materials and Methods section. Lane 1 (panel A) shows the DNA EcoR I Hind III digest. The molecular weight standards shown in panel B are: ladder (lane 1), *S. cerevisiae* chromosomes (lane 2) and *S. pombe* chromosomes (lane 3). The gels shown in A and B are representative of three experiments with similar outcomes.

The next series of experiments were performed with the aim of gaining new insights as to the mechanism(s) involved in the apoptotic response triggered by BHT.

We first examined whether the effects of BHT were a direct consequence of its antioxidant activity and for this purpose analyzed whether other antioxidants were also inducers of apoptosis in U937 cells. This possibility appears to be unlikely since the growth of U937 cells in the presence of *N,N'*-diphenyl-1,4-phenylenediamine (DPPD), Trolox or α-tocopherol did not lead to significant morphological changes (not shown), nor did it produce detectable secondary DNA fragmentation (Table I).

The role of free radical intermediates and/or non-radical electrophilic quinones generated via cytochrome P450-dependent metabolism was subsequently investigated. The results reported in Table II indicate that the extent of secondary

TABLE I BHT, unlike other antioxidants, induces secondary DNA fragmentation

Treatment[a]	DNA fragmentation (%)[b]
Control	5.5 ± 0.7
200 μM BHT	62.8 ± 2.1*
10 μM DPPD	6.7 ± 1.3
1 mM Trolox	6.6 ± 1.7
50 μM α-tocopherol	5.8 ± 1.4

[a]Cells were treated for 3 h.
[b]Secondary DNA fragmentation was measured with the filter-binding assay as described in the Materials and Methods section.
Results are the mean ± SEM of three separate experiments, each performed in duplicate.
*$P < 0.0001$ vs untreated cells (unpaired t test).

DNA fragmentation caused by 100 μM BHT was remarkably increased by the cytochrome P450 inhibitor SKF-525A. Metyrapone, while significantly increasing the level of BHT-induced DNA fragmentation, was much less effective than SKF-525A. Importantly, DNA fragmentation

TABLE II The effect of cytochrome P450 inhibitors on BHT-induced secondary DNA fragmentation

Treatment[a]	DNA fragmentation (%)[b]
Control	5.5 ± 0.3
50 μM SKF-525A	5.9 ± 0.4
100 μM SKF-525A	6.2 ± 0.7
250 μM metyrapone	4.8 ± 1.1
100 μM BHT	13.1 ± 1.6
100 μM BHT + 10 μM SKF-525A	30.2 ± 1.3*
100 μM BHT + 25 μM SKF-525A	37.4 ± 3.4**
100 μM BHT + 50 μM SKF-525A	45.8 ± 3.4**
100 μM BHT + 100 μM SKF-525A	85.3 ± 2.1**
100 μM BHT + 100 μM metyrapone	15.2 ± 1.4
100 μM BHT + 250 μM metyrapone	24.2 ± 1.8*

[a]Cells were exposed for 3 h to 100 μM BHT in the absence or presence of different concentrations of cytochrome P450 inhibitors.
[b]Secondary DNA fragmentation was measured immediately after treatments with the filter-binding assay. Results are the mean ± SEM of three separate experiments, each performed in duplicate.
*$P < 0.05$ and **$P < 0.01$ compared with BHT alone by analysis of variance followed by Dunnett's test.

TABLE III The effects of catalase, AT, BSO, *o*-phenanthroline or NAC on BHT-induced secondary DNA fragmentation

Treatment[a]	DNA fragmentation (%)[b]
Control	5.9 ± 0.8
100 U/ml catalase	6.1 ± 0.9
10 mM AT	5.7 ± 0.7
10 μM BSO	5.8 ± 0.9
150 μM BHT	30.5 ± 4.2
150 μM BHT + 100 U/ml catalase	31.6 ± 5.2
150 μM BHT + 10 mM AT	65.7 ± 5.3**
150 μM BHT + 10 μM BSO	54.6 ± 3.4*
150 μM BHT + 50 μM *o*-phenanthroline	9.6 ± 1.4**
150 μM BHT + 10 mM NAC	7.3 ± 1.2**

[a]Cells were treated for 3 h with 150 μM BHT in the absence or presence of catalase, *o*-phenanthroline or NAC, or following a 6 or 18 h preincubation with AT or BSO, respectively.
[b]Secondary DNA fragmentation was measured with the filter-binding assay.
Results are the mean ± SEM of three separate experiments, each performed in duplicate, and were significantly different from those for the DNA fragmentation induced by BHT alone at *$P < 0.01$, **$P < 0.001$ (unpaired t test).

was not detected in cells exposed to SKF-525A or metyrapone alone. Thus, the BHT-derived products responsible for the apoptotic response are different from those resulting from cytochrome P450 metabolism.

Additional experiments were performed to assess whether the process of BHT-induced apoptosis was associated with an altered redox state. We found that pharmacological depletion of catalase or soluble thiols significantly increased the extent of secondary DNA fragmentation caused by BHT (Table III). In these experiments the cells were exposed to either 10 mM 3-amino-1,2,4-triazole (AT) (6 h) or 10 μM L-buthionine-[S,R]-sulfoximine (BSO) (18 h) and then treated for 3 h with 100 μM BHT. Exposure to AT promoted an 82.9% decrease in catalase activity (untreated cells, 7.13 ± 0.2 Sigma units/mg protein) and growth in BSO led to an 80% decrease in non-protein thiols (untreated cells, 30 ± 1.5 GSH equivalents/mg protein). Since addition of extracellular catalase (100 U/ml) did not affect the DNA fragmentation triggered by BHT, the above results strongly suggest that the apoptotic response is associated with an altered redox state that was at least in part mediated by the intracellular formation of hydrogen peroxide. Consistent with this notion are the results indicating that the BHT-induced DNA fragmentation is either markedly reduced or suppressed by the iron chelator *o*-phenanthroline or the thiol compound *N*-acetylcysteine (NAC), respectively. Importantly, DNA fragmentation was not detected in cells exposed to AT, BSO, *o*-phenanthroline or NAC alone. Finally, reagent hydrogen peroxide was a potent inducer of apoptosis in U937 cells (not shown).

DISCUSSION

The present study was performed to determine the mode of U937 cell death caused by the antioxidant BHT. We report that this response (Figure 1) was preceded by the appearance of typical morphological and biochemical changes that are commonly associated with apoptosis. In particular, BHT was found to cause extensive cell blebbing (Figure 2) as well as secondary DNA

fragmentation (Figure 3). The formation of oligonucleosomal DNA fragments (Figure 4A) and of ≤ 50 kb double-stranded DNA fragments (Figure 4B), two specific markers of the apoptotic response,[25] was also detected by conventional gel electrophoresis and by PACE, respectively.

Thus, our results demonstrate that BHT induces apoptosis in U937 cells. We also provided experimental evidence suggesting that this response is not causally linked to the antioxidant properties of BHT since the other tested antioxidants DPPD, Trolox and α-tocopherol did not induce apoptosis in U937 cells (Table I).

The finding that cytochrome P450 inhibitors, and in particular SKF-525A, provoke a dramatic enhancement in the BHT-induced apoptotic response (Table II) suggests that the latter event is negatively controlled by the cytochrome P450 monooxygenase system. As a consequence, this metabolic pathway would be expected to promote the formation of species which do not produce apoptosis and concomitantly lower the intracellular fraction of BHT available for the formation of species mediating the apoptotic response. In addition and/or alternatively, the metabolites generated via the cytochrome P450 pathway may act as inhibitors of the apoptotic response. It is important to point out, however, that our findings are entirely based on studies using inhibitors and the possibility that these agents exert their effects via a mechanism not depending on inhibition of cytochrome P450 cannot be ruled out. Thus, while the above results provide a clearcut indication that the BHT-mediated apoptotic response is not caused by products derived from cytochrome P450 metabolism, further studies are necessary to correctly interpret the biological significance of the enhancing effects promoted by the cytochrome P450 inhibitors.

In the present study we also provided experimental evidence indicating that the apoptotic response evoked by BHT was augmented by depletion of catalase or of soluble thiols and prevented by either *o*-phenanthroline or NAC (Table III). These findings demonstrate that catalase and reduced glutathione, which represents approximately 90% of the NPSH pool, are important constituents of the cellular defence system opposing the apoptosis induced by BHT, and are therefore consistent with the possibility that an altered redox state and hydrogen peroxide generation are two major determinants involved in this response. Importantly, hydrogen peroxide is a potent inducer of apoptosis in U937 cells (not shown). It was recently demonstrated that hydrogen peroxide can be generated via oxidation of BHT catalyzed by a copper-redox cycle mechanism.[27] Since BHT was shown to promote mitochondrial damage in isolated hepatocytes,[19,28] hydrogen peroxide may also be formed via dismutation of superoxide anions generated by the mitochondrial respiratory chain.

In conclusion, this study leads to the novel demonstration that BHT induces apoptosis in U937 cells via a non-antioxidant mechanism and provides experimental results indicating that the apoptotic response is not caused by products of cytochrome P450 metabolism. BHT-induced U937 cell apoptosis appears to be mediated by an altered redox state and hydrogen peroxide.

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