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t-BUTYL-4-HYDROXYANISOLE AS AN INHIBITOR OF TUMOR CELL RESPIRATION

ELIZABETH FONES,* HANNELORE AMIGO,* KIMIE GALLEGOS,* ANIBAL GUERRERO† and JORGE FERREIRA*‡

Departments of †Experimental Medicine and of *Biochemistry and Chemistry, Faculty of Medicine, Universidad de Chile, Santiago, Chile

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Abstract—The effect of *t*-butyl-4-hydroxyanisole (BHA), a widely used food antioxidant additive, on the culture growth, oxygen consumption, and redox state of some electron carriers of intact TA3 and 786A ascites tumor cells has been studied. BHA inhibited culture growth and respiration of these two tumor cell lines, by inhibiting the electron flow through the respiratory chain. Experiments to determine its site of action showed that BHA did not inhibit noticeably the electron flow through cytochrome oxidase, due to the ability of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine to bypass the BHA inhibition of the respiration. Electron flow through the ubiquinone-cytochrome b–c_1 complex also was unaffected by BHA; in fact, BHA failed to inhibit the oxidation of duroquinol. Spectrophotometric experiments are in accordance with studies carried out using synthetic electron donors. The redox state of $NAD(P)^+$, determined in steady-state conditions, changed to a more reduced level, and the redox states of ubiquinone, cytochrome b, cytochromes $c + c_1$ and cytochromes $a + a_3$ changed to a more oxidized level. These observations suggest that the electron transport in the tumor mitochondria was inhibited by BHA at the NADH-dehydrogenase–ubiquinone level (energy-conserving site 1). These findings could explain, in part, the cytotoxic effect of BHA.

3(2)-*t*-Butyl-4-hydroxyanisole (BHA) is perhaps best known for its activity as an antioxidant of relative low toxicity to mammalians; therefore, it has been used widely as a human and animal food additive since 1947 [1]. BHA has toxic effects at extremely high doses, having an LD_{50} on the order of 2000 mg/kg when administered by stomach tube to most animals; at a level of 50 mg/kg/day it appears to be free of any obviously injurious effects [1–4]. Moreover, it has been demonstrated that BHA is a versatile inhibitor of neoplasia induced by various types of chemical carcinogens. When it is given prior to and/or simultaneously with certain carcinogens. BHA inhibits chemical carcinogenesis in various organs of rats or mice [4–8]. Induction of drug-metabolizing enzymatic systems that may interrupt the neoplastic process could explain, in part, this anticarcinogenic effect. Thus, BHA enhances the hepatic and peripheral activities of several detoxificating enzymes, including glutathione-*S*-transferase, UDP-glucuronyltransferase, NAD(P)H:quinone reductase, epoxide hydrolase, glucuronide-conjugating systems and NADPH oxidase activity. It also raises the levels of glutathione and enzymes concerned with its reduction [7–11]. In addition, there is a decrease in plasma catalase and peroxidase activities and in the oxygen uptake of liver from rats fed with 0.01–0.1% BHA, using succinate as a substrate, whereas the liver from rats fed with 0.1–1.0% BHA have increased oxygen uptake and altered oxidative phosphorylation [3]. Because of the complexity of the response of BHA, it is difficult to establish which of the biochemical alterations, or which combination of effects, is critical for reduction in the carcinogenic properties of several chemicals in animals fed with BHA [6].

Recently, Piccardo *et al.* [12] reported that BHA and other phenolic compounds inhibit the growth of several human tumor cell lines in culture. The mechanisms of this toxic effect of BHA have not been elucidated. A direct interaction between BHA and some vital enzymatic system could occur. Since BHA inhibits the growth and oxygen consumption of trypanosome parasites as well as the respiration of rodent tumor cells [13, 14], we examined whether the cytotoxic effects of BHA are also associated with the respiratory chain of tumor cells. In this report, it is shown that BHA inhibited the growth and the mitochondrial electron transport of intact cells between the NADH dehydrogenase and ubiquinone.

MATERIALS AND METHODS

Chemicals. Fetal calf serum was purchased from Gibco Laboratories (Santa Clara, CA). Glutamine, rotenone, antimycin, Dulbecco's modified Eagle's medium, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), duroquinone, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), KCl, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2-amino-2-hydroxymethyl-1,3-propanediol-HCl (Tris–HCl) together with the following phenolic antioxidants: BHA, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), nordihydroguaiaretic acid (NDGA), 2′,4′,5′-trihydroxy-butyrophenone (THBP) and 4,4′-isopropylidenediphenol (IPDP) were purchased from Sigma Chemical Co. (St Louis, MO). The stock solutions of these antioxidants were

‡ Correspondence: Dr Jorge Ferreira, Departamento de Bioquímica y Química, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago-7, Chile.

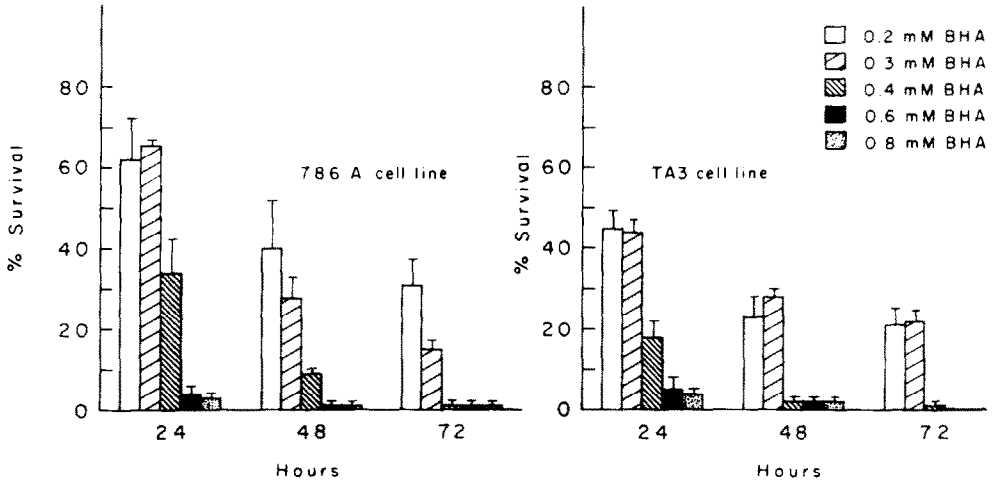

Fig. 1. Percentage of survival of 786A and TA3 cell lines cultured for periods up to 72 hr in the presence of BHA. Each result is the mean of two to four experiments with each assay performed in quadruplicate.

prepared in ethanol or dimethyl sulfoxide (DMSO); no effects of these solvents at the concentrations used in our experiments were observed. NaCl was obtained from May & Baker (Dagenham, U.K.). Duroquinol was prepared from duroquinone in alcoholic solution by reduction with sodium borohydride, was recrystallized, and the stock solution was dissolved in DMSO. All other reagents were of the highest purity commercially available.

Harvesting of tumor cells. The following ascites tumors were grown by weekly intraperitoneal injection into the appropriate tumor bearing-mice: the 786A ascites tumor was carried in young adult male Swiss mice and the TA3 ascites tumor was propagated in young adult male CAF 1 Jax mice. All animals were fed with a standard laboratory chow and water *ad lib*. The tumor cells were harvested 7–9 days after intraperitoneal inoculation of ascites fluid from donor mice by centrifugation at 100 *g* for 5 min at 4° and were washed twice with 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCL, pH 7.4, essentially as described by Moreadith and Fiskum [15]. The tumor cells were resuspended in the same medium at a concentration of 30–35 mg of protein/ml. The cells appeared to be virtually free of erythrocytes and other contaminants and showed a viability of 95–98% as indicated by exclusion of trypan blue determined by microscopy. The protein concentration was determined by a modified biuret reaction standardized with serum albumin [16]. Both cell lines were cultured in the absence or presence of BHA in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 25 mM Hepes, 44 mM $NaHCO_3$ and penicillin (100 units/ml) and streptomycin (100 μg/ml). For the experiments, 1.2–1.5 × 10^5 cells/ml were seeded in 30 ml of culture medium, using 100-ml culture flasks and grown at 37° for up to 96 hr. The cells were allowed to grow for 24 hr, and then BHA was added. Cell numbers were determined with a Neubauer counting chamber every 24 hr.

Cell respiration. Oxygen uptake was measured polarographically at 25° with a Clark electrode No. 5331 (Yellow Springs Instrument) and using a YSI model 53 monitor linked to a 100 mV monochannel

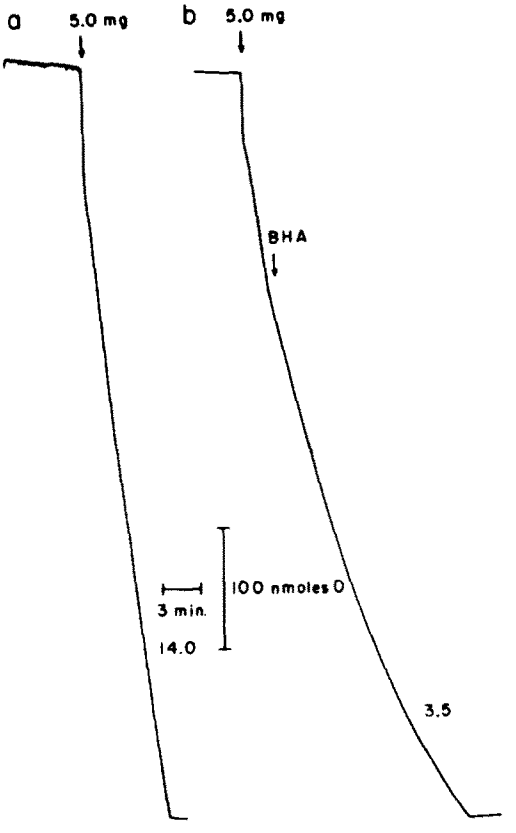

Fig. 2. Effect of BHA on the respiration of the TA3 ascites tumor cell line. In a and b, 5 mM glutamine was used as substrate. Addition of cells (5.0 mg of protein in a and b) and BHA (0.98 μmol in b) was made at the points indicated by the arrows. The number placed above the curves indicate the rate of oxygen consumption/min/mg protein. For other details, see Materials and Methods.

RE 511 recorder. The 2.0-ml reaction mixture contained 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4, plus 5 mM glutamine as substrate and 2.5 mg of protein/ml of ascites tumor cells.

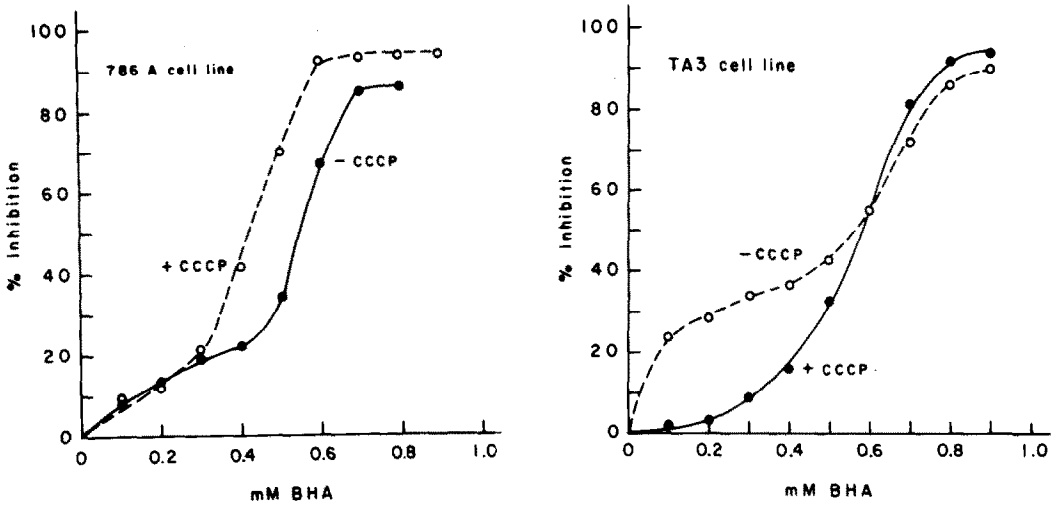

Fig. 3. Effect of BHA concentration on the respiration of 786A and TA3 cell lines in the presence and absence of the uncoupler CCCP. Results are expressed as percent inhibition of the oxygen consumption in the absence of inhibitor; control activities were 13.7 (0.07 μM CCCP) and 11.8 (−CCCP), and 21.1 (0.07 μM CCCP) and 15.5 (−CCCP) nmol 0/min/mg protein in 786A and TA3 cell lines respectively. The inhibitory effects of different BHA concentrations were obtained from experiments like those of Fig. 2. Each point is the mean of four to six independent experiments. Other conditions were as in Fig. 2.

Spectrophotometric determinations. The redox state of the respiratory carriers was monitored at 25° by dual-wavelength spectrophotometry (Aminco DW-2). The 2.5-ml reaction medium consisted of 150 mM NaCl, 5 mM KCl, 10 mM Tris–HCl (pH 7.4) and 5 mM glutamine as substrate. The wavelength couples used were: 340–390 nm for $NAD(P)^+$ [13], 275–245 nm for ubiquinone [17], 430–410 nm for cytochrome *b* [18], 550–540 nm for cytochromes $c + c_1$ and 445–455 nm for cytochromes $a + a_3$ [17]. The cuvette with a 1.0-cm light path was provided with magnetic stirring. The addition of BHA, as indicated in the figures, was made by rapid injection from microsyringes in such a way as to achieve the shortest possible mixing time.

RESULTS

We have reported previously that BHA is an inhibitor of the respiration of tumor cells [14]. To establish more precisely the mechanism of action of BHA, we assayed the effect of the antioxidant on the growth, oxygen consumption, and redox states of some electron carriers of tumor cell lines. Figure 1 shows the effects in culture of different concentrations of BHA on the percentage survival of 786A and TA3 ascites tumor cell lines exposed for periods up to 72 hr. All the concentrations of BHA strongly inhibited the growth of both ascites tumor cell lines. For each concentration of BHA tested, toxicity increased with the time of exposure to the chemical, up to 72 hr. At 96 hr of exposure, practically no surviving cells were found in the presence of 0.4 to 0.8 mM BHA, and less than 20 and 10% in cultures with 0.2 and 0.3 mM BHA, respectively (control cultures 96 hr old were used as the standard). No noticeable variation was found in the levels of survival of cells for each BHA concentration tested between the two cell lines used.

A representative polarographic trace of the respiratory rate of the TA3 cell line is illustrated in Fig. 2a. The addition of BHA produced an inhibition of the rate of oxygen consumption (Fig. 2b). The effects of BHA concentration on the respiratory rate of 786A and TA3 cell lines are shown in Fig. 3. Biphasic inhibitory curves were observed when the BHA concentration was increased, which is better perceived on the respiratory rate of the TA3 cell line. Inhibition increased with the addition of small amounts of BHA up to 0.1 mM. Between 0.1 and 0.4 mM BHA, the inhibition increased slightly, only to 22 and 36% for the respiratory rates of the 786A and TA3 cell lines respectively. Further increases in BHA concentrations resulted in a concentration-dependent inhibition. Maximal inhibition (85–90%) of the respiratory rates of 786A and TA3 cell lines was attained at about 0.8 and 0.9 mM respectively; the I_{50} was 0.55 mM for the former and 0.6 mM for the latter. In the presence of the uncoupler, however, sigmoidal inhibitory curves were observed. Maximal inhibition (92–94%) of CCCP-stimulated oxygen consumption by the 786A and TA3 cell lines was obtained, respectively, at about 0.6–0.8 mM and 0.8–0.9 mM. The I_{50} values were about 0.43 and 0.58 mM for the 786A and TA3 cell lines. Only slim variation in the level of inhibition was found among these two tumor cell lines. These results suggest that the level of inhibition, although depending on the inhibitor concentration, is not predominantly dependent on whether the oxygen consumption is coupled or uncoupled to the ADP phosphorylation. Consequently, the primary interaction occurs in the electron transfer pathway of the mitochondrial respiratory chain.

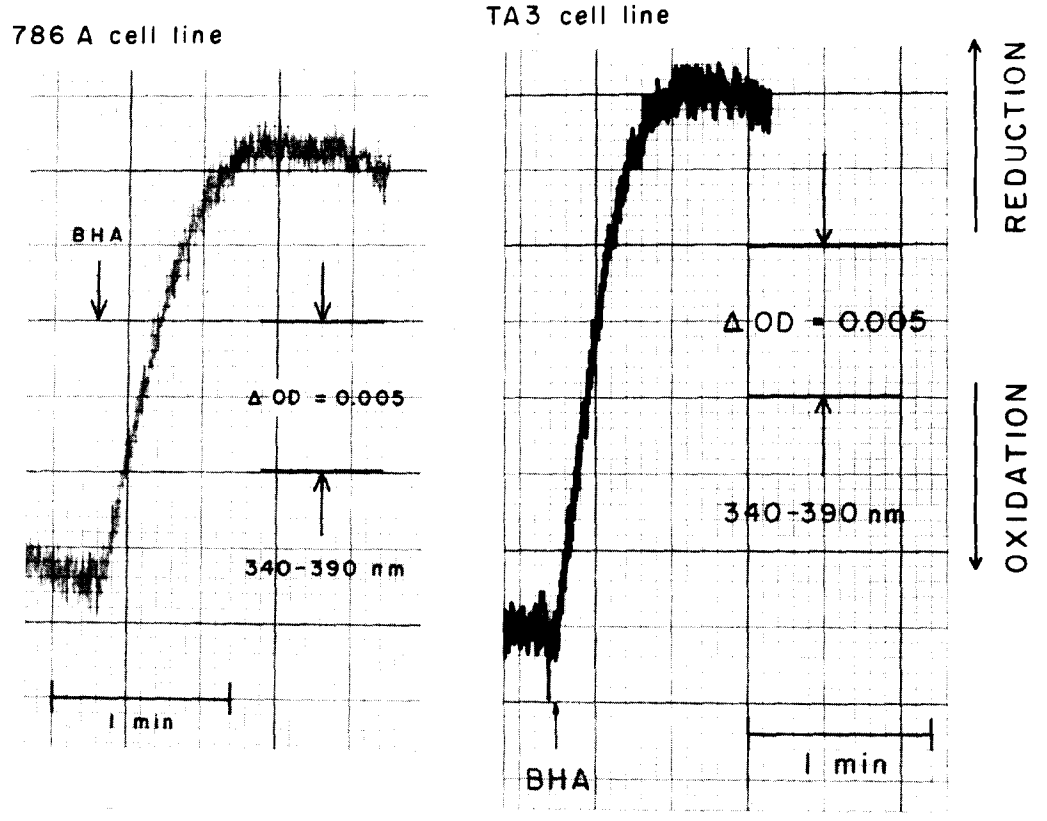

Fig. 4. Effect of BHA on the redox state of NAD(P)$^+$ in 786A and TA3 cell lines. Tumor cell lines (12.5 mg protein) were preincubated at 25°. The reduction was initiated by the addition of 2.5 μmol BHA. For other experimental conditions, see Materials and Methods.

To establish the inhibitory site of BHA within the mitochondrial electron transport chain of intact tumor cells, two different experimental approaches were employed. In the first, we studied the capacity of BHA to change the redox state of some electron carriers. Figure 4 shows the redox state of NAD(P)$^+$ in 786A and TA3 tumor cells. When BHA was added, NAD(P)$^+$ was partially reduced as indicated by the upward deflection of the 340–390 nm traces. BHA changed the redox levels of ubiquinone and cytochrome *b* towards more oxidized states (Figs 5 and 6), both in 786A and on TA3 tumor cells, as indicated by the upward deflection of the 275–245 nm trace and the downward deflection of the 430–410 nm trace respectively. The magnitude of the changes of redox state is dependent on the BHA concentration and the amount of tumor cells. Also, the redox states of cytochromes $c + c_1$ and $a + a_3$ were shifted to more oxidized states (Fig. 7). Experiments carried out adding rotenone showed results similar to those obtained with BHA. These results suggest that the primary site at which BHA inhibited the respiratory chain was located before ubiquinone.

A second type of experiment was performed to determine the effect of BHA on oxygen consumption when well characterized synthetic substrates, donating electrons to the energy-conserving sites 2 and 3 of the respiratory chain, were added. The effect of BHA on the electron flow through site 3, i.e. cytochrome oxidase activity, was determined by assessing the ability of TMPD to bypass the inhibition of the respiration caused by BHA. This approach is based on the principle that NADH is nonenzymatically oxidized by TMPD, which in turn feeds electron back to cytochrome *c*, permitting the cytochrome oxidase reaction [19, 20]. The traces presented in Fig. 8 show that the inhibition of the oxygen consumption by rotenone, antimycin and BHA was bypassed by TMPD. These findings demonstrate that the site of inhibition of electron transport by BHA is not at the cytochrome oxidase step. The most direct test of the action of BHA on the energy-conserving site 2, i.e. the cytochrome b–c_1 complex, was made by using duroquinol as the electron donor to site 2 of the respiratory chain [21, 22]. Figure 9 shows that the inhibition of the oxygen uptake by rotenone and BHA was reversed by duroquinol but not in the presence of antimycin. These experiments not only support the conclusion that BHA inhibits electron flow at some point before cytochrome *b*, but they also indicate that BHA does not inhibit electron flow from cytochrome *b* to oxygen.

Thus, these two types of experiments show not only that BHA did not perceptibly inhibit electron flow through sites 2 and 3 of the respiratory chain, i.e. the CoQ–c_1 span and cytochrome oxidase, respectively, but also that the predominant inhibitory site of BHA is located at some point before CoQ.

Table 1 summarizes the effects of various phenolic antioxidants on the respiration of tumor cells in the

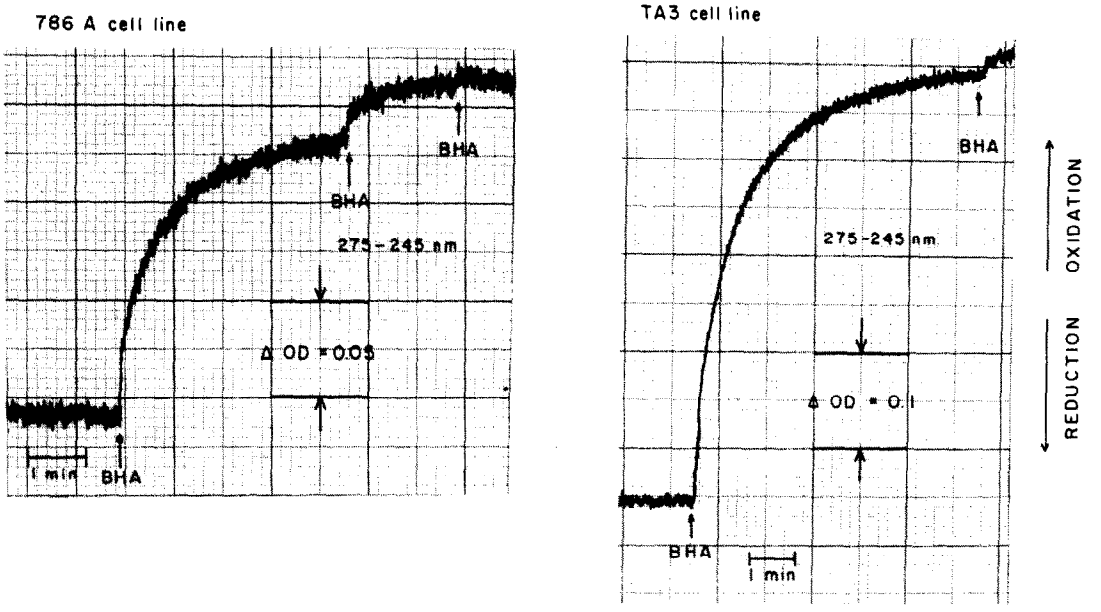

Fig. 5. Effect of BHA on the redox state of ubiquinone in 786A and TA3 cell lines. Tumor cell lines (2.0 mg protein) were preincubated at 25°. The oxidation was initiated by the addition of 2.0 μmol BHA. For other experimental conditions, see Materials and Methods.

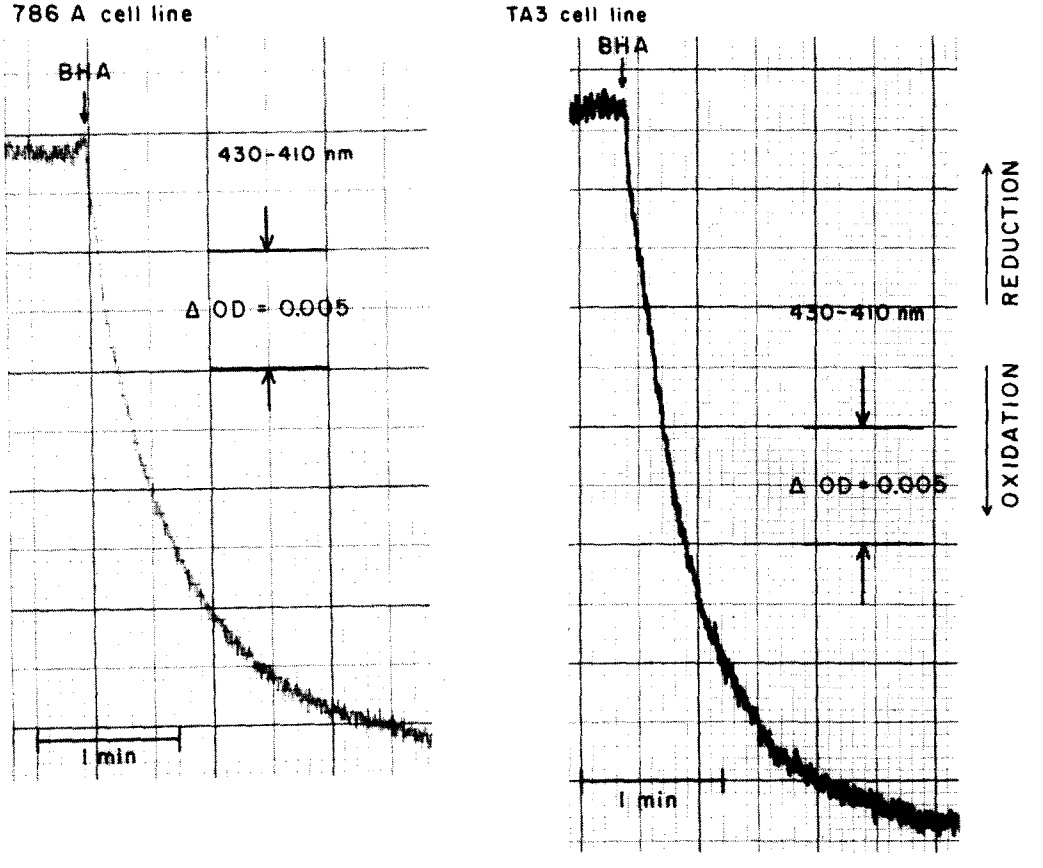

Fig. 6. Effect of BHA on the redox state of cytochrome *b* in 786A and TA3 cell lines. At the points indicated by the arrows, 2.5 μmol BHA was added to 786A (13.0 mg protein) and TA3 (8.0 mg protein) cell lines.

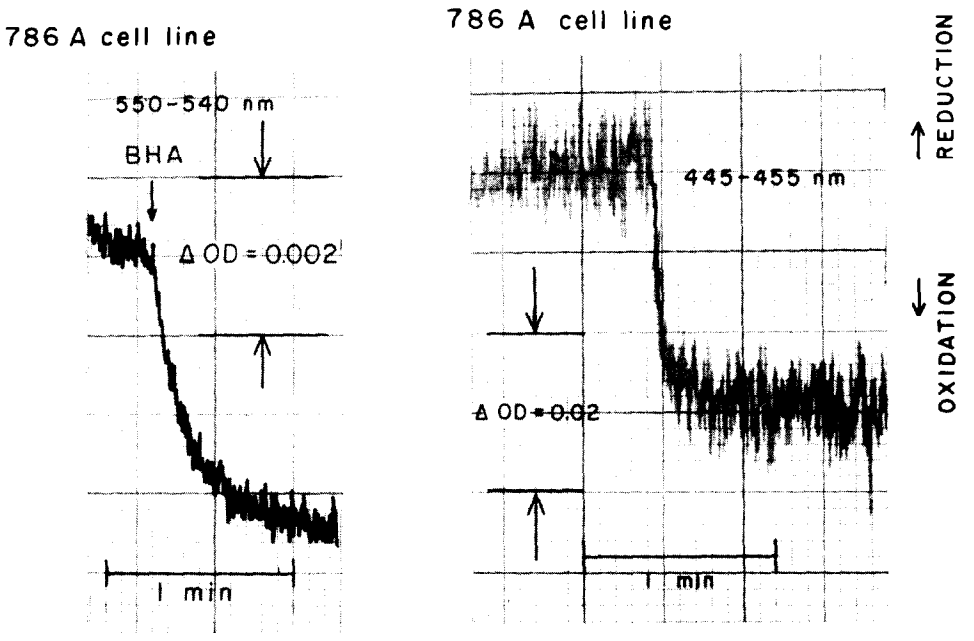

Fig. 7. Effect of BHA on the redox state of cytochrome *c* and cytochrome $a + a_3$ in the 786A cell line. At the points indicated by the arrows, 3.0 μmol BHA was added to 12 and 14 mg protein of the 786A cell line respectively.

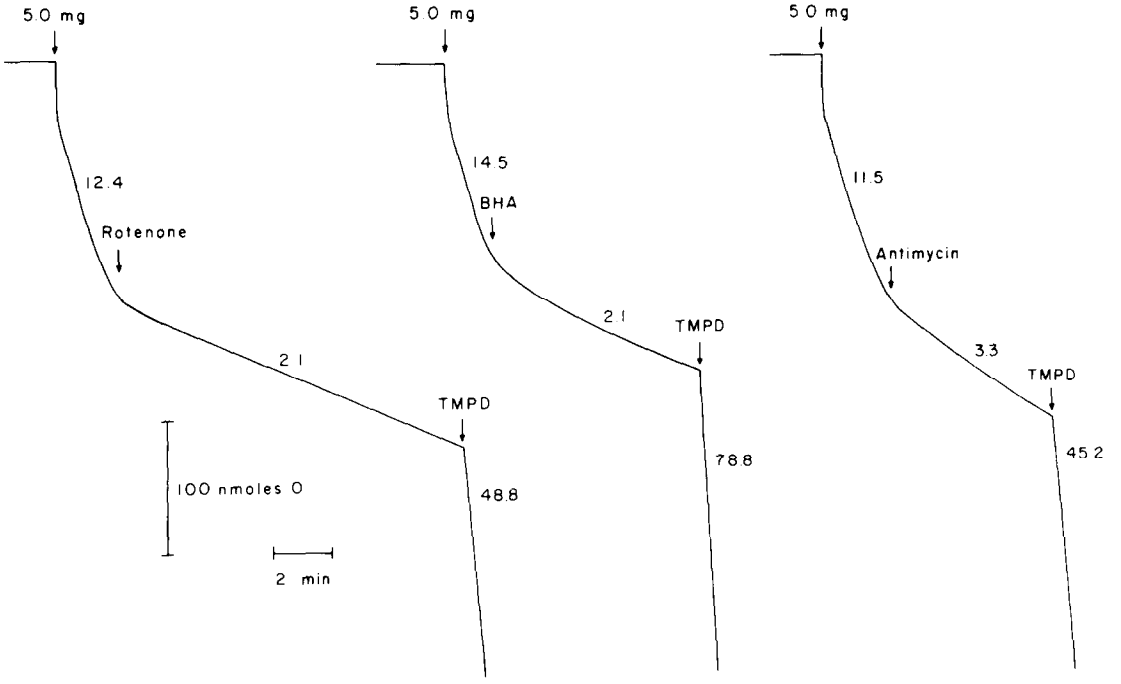

Fig. 8. Effect of BHA on 786A cell respiration via TMPD bypass. At the points indicated by the arrows, 786A (5.0 mg), rotenone (0.4 nmol), antimycin (0.05 μg/mg protein), BHA (1.4 μmol) and TMPD (3.0 μmol) were added. The numbers placed above the traces indicate the rate of oxygen consumption/min/mg protein. For other details, see Materials and Methods.

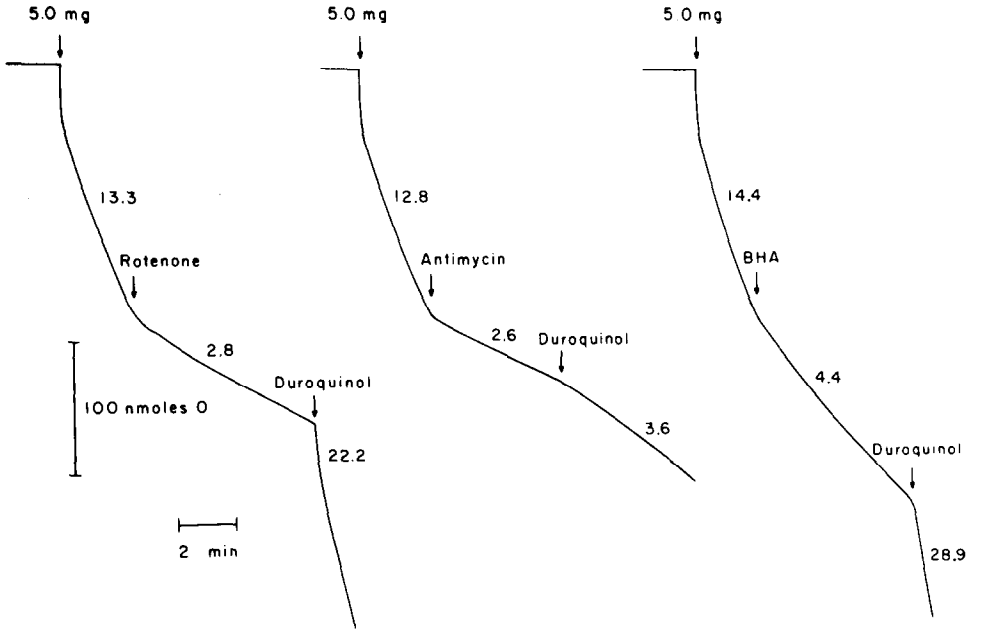

Fig. 9. Effect of BHA on the rate of duroquinol oxidation by 786A tumor cells. The concentration of duroquinol was 1.4 mM and that of BHA 0.8 mM. Other experimental conditions were as given in the legend of Fig. 8.

Table 1. Effects of phenolic antioxidants on tumor cell respiration

	I_{50} (mM)			
	TA3 Cell line		786A Cell line	
Antioxidant	−CCCP	+CCCP	−CCCP	+CCCP
BHT	NI*	NI	NI	NI
NDGA	0.21	0.20	0.24	0.22
IPDP	0.39	0.42	0.42	0.39
THBP	0.67		0.59	0.48

I_{50} corresponds to the antioxidant concentration needed to inhibit 50% of respiration. Data were calculated from curves of oxygen uptake versus antioxidant concentrations (N = 3–6). See Materials and Methods for experimental details.

* No inhibition observed with up to 5 mM.

presence and absence of the uncoupler CCCP. The concentration of these phenols required to inhibit 50% of the oxygen consumption was about 0.2 to 0.7 mM. NDGA was the most potent inhibitor of all the antioxidants tested. It has been reported that NDGA inhibits anaerobic and aerobic glycolysis and respiration of K-2 ascites cells and L1210 mouse leukemia ascites cell lines [23]. It is also an effective inhibitor of electron flow and energy transfer in isolated mammalian mitochondria [24, 25]. The I_{50} of the oxygen consumption by 786A and TA3 tumor cells was between 0.2 and 0.24 mM NDGA, which is four times higher than what has been reported for rat liver mitochondria [24]. Plausible explanations could be that intramitochondrial NDGA concentration in intact tumor cells is less than in the surrounding medium, that NDGA does not easily enter into the cell at pH 7.4, or that it is bound to other proteins. It has been reported that NDGA inhibits, for example, alcohol dehydrogenase, catalase, and peroxidase activities of different tumor cell lines [23]. No noticeable variation in the I_{50} values of these phenols was found among the different cell lines tested, regardless of whether the respiration was coupled or uncoupled. It is interesting to note that up to 5 mM BHT did not inhibit the respiration; at higher concentrations, precipitation of BHT in the assay medium was observed.

DISCUSSION

Many phenols have been shown to uncouple oxidative phosphorylation in mitochondria [26, 27]. BHT does not inhibit the oxygen consumption of rat liver mitochondria [24]. Similar results have been observed in tumor cell lines tested by us. BHT possesses only weak uncoupling properties [28]. Many other phenols have been shown to inhibit the mitochondrial electron transport chain. The structural configurations of the *o*- or *p*-dihydroxy, *p*-methoxy, and *p*-phenyl configuration of phenolic compounds are important for this reported biological activity [24, 27, 29]. Thus, these phenolic derivatives could participate in reversible conversions to quinone-like structures [8, 23], competing with CoQ for the electrons that are transferred in the respiratory chain [13, 14].

Our observations in this study suggest that BHA, which enters easily into the cells [12], is an inhibitor of the reoxidation of NADH by the mitochondrial respiratory chain of TA3 and 786A ascites tumor cells, since oxygen consumption is prevented by this

compound, both in the presence and absence of CCCP within the same concentration range. Consequently, it would appear that BHA inhibits the various NAD^+-linked substrates. Inhibition curves of coupled oxygen consumption by BHA have a biphasic shape; the first phase (at low BHA concentration) was not observed when the uncoupler was added, which suggests that the drug interferes slightly with the phosphorylation mechanism *per se* or with the adenine nucleotide translocase (Fig. 3). On the other hand, those curves obtained in the presence of CCCP were sigmoidal in nature, thus indicating a cooperative inhibitory effect on the electron flow, which would suggest the existence of more than one site of inhibition. Further experiments in isolated mitochondria or submitochondrial particles will be necessary to elucidate its inhibitory kinetic mechanism. Tests of the effect of BHA on different segments of the respiratory chain show that energy-conserving site 3, the cytochrome oxidase reaction, was not affected (Figs 7 and 8). Similarly, electron flow through the b–c_1 complex of site 2 was not inhibited by BHA, indicated by two different types of experiments and particularly by the failure of BHA to inhibit the oxidation of duroquinol (Fig. 9), suggesting that BHA interferes with various NADH-linked dehydrogenases, substrate transport systems, or with the components before CoQ of the electron transport system.

This compound also caused a change of the redox state of $NAD(P)^+$ towards a more reduced level, as indicated by the upward deflection of the 340–390 nm trace (Fig. 4). Experiments determining the redox states of electron carriers suggest that BHA interferes by inhibiting the energy-conserving site 1 of the respiratory chain i.e. NADH-dehydrogenase, excluding the influence of BHA on both substrate transport systems and NAD^+-linked dehydrogenases. The effect of BHA on other dehydrogenases remains to be elucidated.

Finally, toxicological significance of the damage to mitochondria from TA3 and 786A tumor cells by BHA should be assessed. If it is considered that, in recent years, accumulated evidence indicates that oxidation of glutamine to CO_2, not glucose, is the major energy source for tumor cells even in the presence of physiological levels of glucose [30–32], their mitochondria would be the principal site of oxidative metabolism and ATP synthesis. BHA caused a profound deflection in the electron transport chain at the NADH–CoQ segment, impairing the utilization of NAD^+-linked substrates, which would result in a pronounced reduction of ATP synthesis. Thus, BHA would act as an inhibitor of oxidative phosphorylation, preventing ATP synthesis, which, in turn, would lead to diminished activity of cellular energy-dependent processes. Consequently, BHA acts by inhibiting cultured tumor cell growth, since there is good correspondence between the concentration of BHA that is cytotoxic and the concentration that inhibits mitochondrial respiration. These findings would explain, in part, the antitumoral effect of BHA. However, the BHA concentration which induces cytosolic NAD(P)H:quinone oxidoreductase activity is 0.03 to 0.06 mM [8]. This enzymatic activity is considered to be a measure of induction of several protective enzymes in rodents [8]. Consequently, the anti-carcinogenic action of BHA could be within this same range of concentrations, which is about ten times lower than the range that has a cytotoxic effect.

It may be pointed out that there are suggestive similarities between the action of BHA on the respiration of tumor cells described here and the antineoplastic activity of NDGA [23], both agents being inhibitory at the mitochondrial electron flow. Although it is still premature to consider BHA as an antitumor drug, this possibility could form the basis for further studies.

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