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TITLE: EFFECTS OF BUTYLATED HYDROXYANISOLE ON THE METABOLISM AND MUTAGENIC AND TRANSFORMATION POTENTIALS OF BENZO(a)-PYRENE IN CULTURED MAMMALIAN CELLS

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EFFECTS OF BUTYLATED HYDROXYANISOLE ON THE METABOLISM AND MUTAGENIC AND TRANSFORMATION POTENTIALS OF BENZO[a]PYRENE IN CULTURED MAMMALIAN CELLS.

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INTRODUCTION

The addition of the antioxidant butylated hydroxyanisole (BHA) to diets containing the carcinogen benzo(a)pyrene (BaP) showed a pronounced suppression of neoplasia of the lung and forestomach in rodents (22,23). A number of *in vitro* studies indicate that antioxidants inhibit microsome- or S9-mediated BaP mutagenicity in the Ames *Salmonella* test (3,6,15,18). It has also been reported that antioxidants inhibit Syrian hamster embryonic (SHE) cell-mediated BaP mutagenicity in V79 cells (10).

The mechanism(s) by which BHA inhibits BaP-induced neoplasia *in vivo* and mutagenicity *in vitro* has been the subject of a number of investigations. Incubation of BaP with microsomes isolated from various rodent species indicates that BHA treatment results in (a) less binding of BaP metabolites to added DNA (20); (b) reduction in hepatic microsomal mixed function oxidase activity resulting in a decrease of Bap hydroxylation (18,25); and (c) a decrease in epoxidation of BaP with enhanced formation of 3-hydroxy-BaP (11). The addition of BHA to the diets of mice inhibits BaP-DNA adduct formation in their forestomachs (1,13). BHA also appears to increase the activity of conjugating enzymes such as glutathione-S-transferase and UDP-glucuronyl transferase (3,4,7). Furthermore, BHA appears to induce elevated levels of tissue glutathione, and an increase in the activities of epoxide hydratase and glucose-6-phosphate dehydrogenase (5,7). These observations indicate that this antineoplastic and antimutagenic agent broadly affects the processes involved in metabolic activation and detoxification of BaP. Owing to the complexity of these BHA effects, the precise mechanisms of action would be difficult to resolve from *in vivo* studies alone.

In the present study we have examined the effect of BHA on BaP metabolism in SHE cells. In addition, a SHE cell-mediated Chinese hamster ovary (CHO) cell mutagenesis

and a SHE cell transformation assay have been used to study the biological effects of BHA. Our results indicate that BHA reduces the metabolism of BaP in SHE cells, suppresses BaP-induced mutagenicity in target CHO cells and reduces morphological transformation by BaP in SHE cells. The application of these mammalian in vitro assays should facilitate both the identification of other protective agents and reveal the mechanisms of their anticarcinogenic effects.

MATERIALS AND METHODS

Cells and Culture Conditions

Chinese hamster ovary cells (CHO-AA8-4) were cultured under conditions described elsewhere (21). Briefly, cells were grown in suspension culture at 37°C in alpha-MEM medium (GIBCO) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% fetal calf serum (FCS) (v/v, Flow Laboratories, Inc.). Colony plating assays, to determine plating efficiency and mutation selection, were done in 60- and 100-mm dishes. The dishes were incubated at 37°C in a humidified CO_2 incubator (maintaining about 5% CO_2 tension).

Primary Syrian hamster embryonic (SHE) cultures were prepared by trypsinization of 13-day-old fetuses of randomly bred Syrian hamsters (from Eagle Laboratory, Farmersburg, IN). The primary cultures were seeded at $1x10^7$ cells per 75 cm^2 flask in IBR modified Dulbecco's Eagles reinforced medium (BioLabs, Northbrook, IL.) containing 20% heat inactivated FCS and incubated at 37°C in an atmosphere of 7.5% CO_2. Near confluent cultures were then trypsinized and stored in liquid nitrogen. Secondary cultures of these cryopreserved cultures were tested with BaP for their ability to undergo morphological transformation. Those cultures giving positive responses were used in experiments designed to measure carcinogen metabolism, mutagenesis in CHO, and morphological transformation. Those cultures which were not responsive to BaP were used to support cell growth in transformation assays (these cells are also referred to as a "feeder layer" but should not be confused with cells used in mutagenesis assays).

Mutagenesis Assay:

Mutagenesis assays were performed as described by Huberman and Sachs (9) with some modifications.

Cryopreserved primary SHE cells were rapidly thawed, seeded into 75 [illegible] flasks, and incubated at 37°C for three days. Feeder layer plates were prepared by first X-irradiating these cells with 4000 r followed by trypsinization and seeding into 60-mm dishes ($2x10^6$ cells/dish). Target CHO cells at a density of $3x10^5$ were then plated onto these feeder layer SHE cells. Unless otherwise stated these cells were immediately treated with BHA for 18 h before the carcinogen (BaP) was added. Following an additional 48 h of treatment the cells were trypsinized and counted using a haemocytometer. CHO cells are easily distinguished from the X-irradiated SHE cells in that the latter are considerably larger in size. Between 200 and 500 cells were plated in 60-mm dishes to determine the surviving fraction. The remaining cells were plated into 100-mm dishes and subcultured every 2 days until an optimum expression period of 8 days had elapsed. The cells were then trypsinized and plated at $2x10^5$ per 100-mm dish in alpha MEM with 10% dialyzed FCS and 10 μM 6-thioguanine (6-TG) (Calbiochem-Behring, Corp.). These plates were incubated at 37°C for an additional 6-8 days at which time colonies of mutant cells were fixed, stained, and enumerated. Plating efficiencies for each dose point were determined at the time of selection of 6-TG resistance in order to quantitate the actual number of innoculated cells that were capable of forming colonies. The mutation frequencies shown have been corrected for plating efficiencies.

Morphological Transformation Assay

Three hundred freshly thawed SHE cells (passage 2) were plated in 4 ml of IBR medium with 20% heat inactivated FCS in a 60-mm dish containing $6x10^4$ X-irradiated feeder cells (4000 r). Chemical treatment was performed as described by DiPaolo (8) and Pienta et al. (17). Chemicals at twice the final concentration in 4 ml of IBR media containing 20% heat inactivated FCS were added to the dishes (also containing 4 ml of medium). Colony morphology was determined with the aid of a stereoscopic microscope. Colonies were scored as non-transformed if they possessed a regularly oriented arrangement of cells and were distinguished from transformed colonies which exhibited three-dimensional growth with random orientation and extensive crossing-over of cells (8,19).

Analysis of BaP Metabolites in the Medium

For the analysis of BaP metabolism in SHE cells,

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X-irradiated cells (4000 r) at $2x10^6$ per 60-mm dish were incubated with 1 μg/ml [3H]-BaP for 22 h. BHA was added either at 0 or 18 hours before BaP treatment. At noted times, one ml of medium was removed and twice extracted with 2.5 volumes of ethyl acetate. The organic extract was vacuum-evaporated and the residue was dissolved in a small amount of glass-distilled methanol. The sample was then eluted with a water-acetonitrile gradient through an ALTEX reverse phase ultrasphere-ODS column (4.5 x 150 mm) using a Beckman model 334 high performance liquid chromatography (HPLC) system. The flow rate was 1 ml per minute and fractions were collected every 15 seconds. The position of the known metabolites of BaP were determined using authentic standards obtained from the NCI Repository at IIT Research Institute (Chicago, IL). The amount of BaP metabolized to "water soluble derivatives" was calculated from the radioactivity in the aqueous phase. The background values were determined for each experiment and were subtracted when calculating the percentage of BaP metabolized by the cells.

RESULTS

Cytotoxicity and Growth Inhibition by BHA in CHO cells.

The toxic properties of BHA were first examined by co-incubation of the chemical with CHO cells over a 48 h period. While no significant differences in colony forming ability was observed for cells treated with 0 to 40 μg/ml BHA, the size of the colonies were consistently smaller for BHA-treated cells (data not shown). The effect of BHA on cell growth rate and saturation density was therefore determined using log phase cells growing in spinner cultures. Figure 1 illustrates the growth rates of these cells in the presence of varying concentrations of BHA. The doubling time for the control cultures in this particular experiment was 13 h. While 10 μg/ml of BHA did not significantly alter the growth of CHO cells, the addition of 30 or 40 μg/ml of the chemical to the culture medium increased the doubling time by 2-fold. In the latter cases the saturation densities were also reduced significantly.

Inhibitory effect of BHA on Mutagenicity Induced by BaP.

Many cells, as CHO, lack the capacity to metabolize procarcinogens. However, competent X-irradiated cells, acting as a feeder layer, can provide these functions under

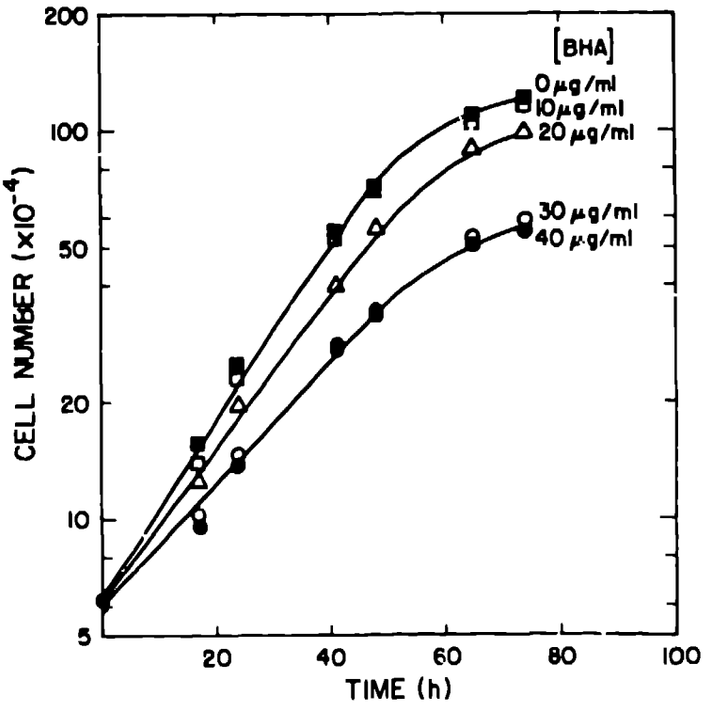

FIGURE 1. Growth inhibition of CHO cells by BHA. CHO cells were treated with BHA at noted concentrations in spinner culture and incubated at 37°C. At various time intervals, cell numbers were determined by Coulter counting.

proper culture conditions (14). Early passage SHE cells when co-cultivated with CHO target cells in the presence of BaP, can elicit a dose-dependent induction of 6-TG resistant mutants in the CHO cells (Figure 2). This particular mutation assay system was used to investigate the effects BHA has on the mutagenic potential BaP (Table I). BHA at 20 μg/ml is itself not mutagenic to CHO cells. The simultaneous addition of both BHA and BaP results in a 50% reduction in the number of 6-TG resistant colonies induced (when compared to BaP treatment alone). If the SHE feeder layer-CHO system is pretreated with BHA for 18 h prior to BaP addition the mutation frequency is reduced even further to 30% of control values. This inhibitory effect by BHA on mutation frequency also remains relatively constant as a function of varying BaP dose (data not shown). These results clearly indicate that BHA can effectively reduce the mutagenic potential of BaP in this assay.

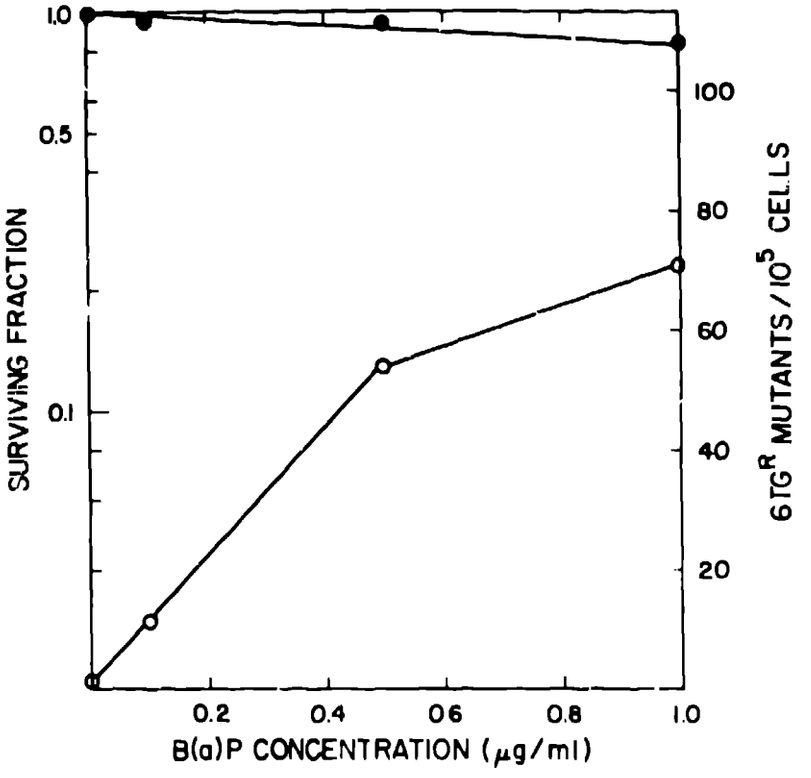

FIGURE 2. Induction of cytotoxicity and 6-TG^R mutational events in CHO cells following SHE cell mediated-metabolic activation of BaP. X-irradiated SHE and CHO cells were treated with BaP for 48 h. Cytotoxicity and mutagenicity measurements were determined as described in Materials and Methods. Open symbols and closed symbols represent mutagenicity and cytotoxicity, respectively.

Inhibitory Effect of BHA on Induced Mutagenesis: Concentration Dependence

The data shown in Figure 3 indicate that the effects of BHA on BaP-induced mutations are concentration dependent. When CHO cells are treated with a fixed level of BaP (1 μg/ml) and varying concentrations of BHA (10 to 40 μg/ml) the inhibition of 6-TG^R mutation induction increases linearly from 20 to 85% (open circles, Fig 3). Pretreatment of the cells with BHA for 18 hours prior to the addition of BaP

results in a similar but more efficient dose-dependent protection from the mutagenic effects of BaP (closed circles). However, cell killing is not affected in the cultures treated with BHA, i.e., BHA does not reduce BaP-induced cytotoxicity.

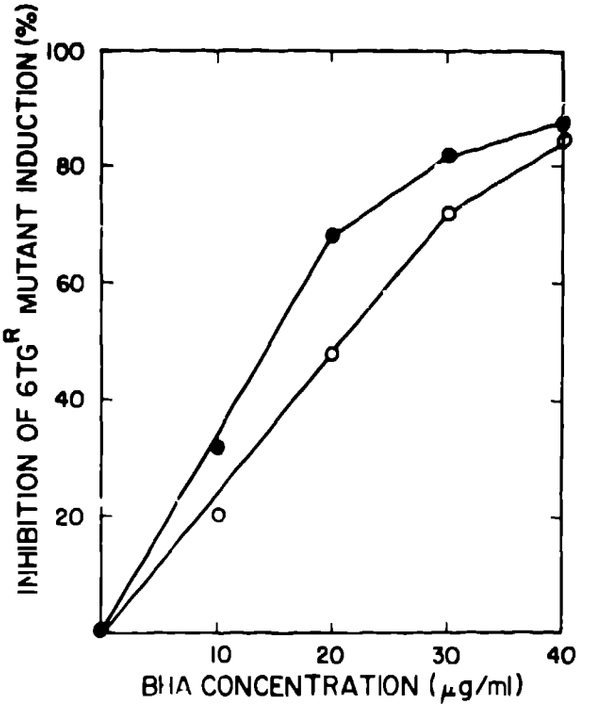

FIGURE 3. Concentration dependence of the inhibitory effect of BHA on BaP-induced mutagenesis. Measurement of 6-TG^R mutants is described in Materials and Methods. Open symbols represent cells treated concomitantly with BaP and BHA. Closed symbols represent cells pretreated with BHA for 18 h before addition of BaP. Inhibition of 6-TG^R mutant induction (IOMI):

$$\text{IOMI (\%)} = 100 \times \left(1 - \frac{\text{Mutation frequency with BHA}}{\text{Mutation frequency without BHA}}\right)$$

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TABLE I.

EFFECT OF BHA ON BaP-INDUCED MUTAGENESIS

BHA (μg/ml)	Cloning Efficiency (%)	6-TG^R Mutants/ 10^5 Cells	Relative Frequency (%)
0	82	71	100
20[a]	78	37	52
20[b]	80	22	31
20[c]	75	1	--

[a] CHO cells were co-cultivated with X-irradiated SHE cells and treated with 20 μg/ml NHA and 1 μg/ml BaP for 48 h.
[b] X-irradiated SHE and CHO cells were pretreated with 20 μg/ml BHA for 18 h. BaP (1 μg/ml) and was then added and the cultures incubated for 48 h.
[c] CHO cells co-cultivated with X-irradiated SHE cells in presence of BHA only.

TABLE II.

EFFECT OF BHA ON BaP-INDUCED MORPHOLOGICAL TRANSFORMATION IN SHE CELLS.

Plating BHA (μg/ml	Efficiency (%)	Transformants Per Survivors	Transformation (%)
0	30	11/905	1.2
5	36	11/1080	1.0
10	34	4/1035	0.3
20	32	3/965	0.3
30	22	0/680	0.0

Inhibition by BHA of Morphological Transformation in SHE Cells Induced by BaP

The inhibitory effects of BHA on the biological properties of BaP are also reflected in the SHE cell morphological transformation assay. SHE cells were treated

with both BHA and BaP for 8 days. The results from both cytotoxicity and morphological transformation determinations are shown in Table II. BHA clearly elicits a dose dependent inhibitory effect on morphological transformations induced by BaP. Concentrations of BHA ranging between 5 and 20 μg/ml effectively reduce the number of SHE cell transformations from 1.0 to 0.3%. No BaP-induced transformants were observed for cells treated with 30 μg/ml BHA, however; cell growth and colony forming ability appeared to be adversely affected under these conditions.

Effects of BHA on SHE Cell-Mediated Metabolism of BaP.

As shown above, BHA dramatically suppresses the mutagenic and transforming potentials of BaP in SHE cell-mediated in vitro bioassays. It is suspected that alterations in the metabolism (oxidation) of BaP or enhancement in the conjugation capacity within the cell or both are the result of the antioxidant treatment (4,5,11,12,18,25). To distinguish between these possibilities we examined the metabolism of BaP in SHE cells treated with BHA. In Figure 4 we show the results of an experiment in which the rates of formation of aqueous-soluble BaP metabolites were determined as a function of the amount of BHA added. After 48 h incubation the amount of water-soluble BaP formed is suppressed approximately 10, 30, 40 and 60% of the control value for 10, 20, 30, and 40 μg/ml of BHA, respectively, added 18 hours prior to the addition of the BaP. When BHA (20 μg/ml) is added concomitantly with the BaP the inhibitory effects of the antioxidant on the water-solubilization of BaP are not seen.

Aqueous-soluble metabolites of BaP formed by hamster cells consist primarily of the non-reactive glucuronide conjugates of monohydroxybenzo(a)pyrene (2,16). The suppression of the mutagenic and transforming potentials in the metabolism of BaP in SHE cells could be related to an amplification of these cells' conjugating capacities by antioxidants. However, it appears that the primary effect of BHA is the reduction of the overall metabolism (oxidation) of BaP itself. This conclusion is based on HPLC analysis of ethyl acetate-soluble metabolites. A series of four HPLC elution profiles are shown in Figure 5. Each

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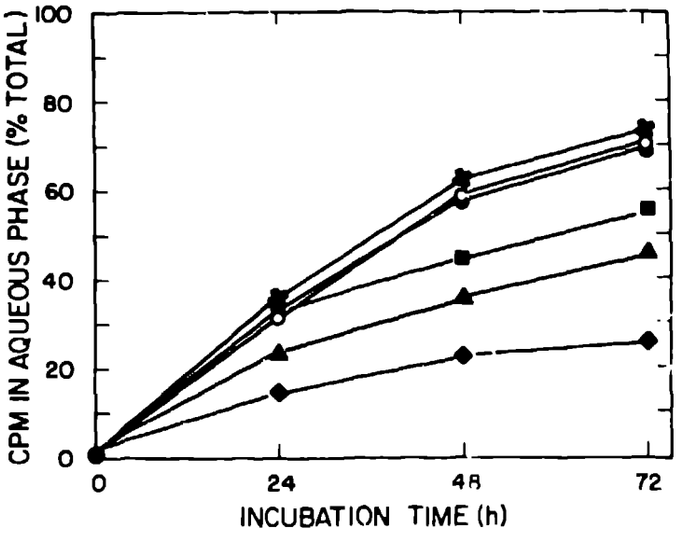

FIGURE 4. Effects of BHA on the metabolism of BaP. X-irradiated SHE cells were incubated with [3H]-BaP at 1 μg/ml. At noted times aliquots of the extracellular medium were removed and the percent of radioactivity which partitioned into an aqueous extraction phase was determined. Symbols used are (✖), control, no BHA added; (●, ■, ▲, and ◆), 10, 20, 30 and 40 μg/ml BHA added, respectively, 18 h prior to BaP addition; and (○), BHA at 20 μg/ml added concomitantly with the BaP.

fraction in each profile is shown as the percent of the total radioactive BaP partitioning into the organic layer. Within 48 h incubation in control, non-BHA treated cultures essentially all the BaP (1 μg/ml) partitioning into the organic layer is metabolized to the 9,10-dihydrodiol, tetrols (hydrolyzed products of diol-epoxides), and extremely polar, undefined compounds. However, after 48 hours incubation in the presence of BHA (20 μg/ml), which was added concomitantly with the BaP, unoxidized BaP is found and a substantial reduction in the amount of tetrols formed is seen. This suppression in metabolism of BaP is seen despite similar levels of BaP water-soluble metabolites formed (see Figure 4). The effect of the antioxidant on metabolism is more vivid if it is added 18 h prior to the addition of the BaP. Normalization of integrated elution peaks to the percent total of material in the complete reaction mix (water plus ethyl acetate layers) indicates a

substantial inhibition by BHA in the metabolism of BaP (e.g., greater than 20% of BaP remains unoxidized after 48 h incubation when BHA is added at -18 h). In addition, it is calculated that tetrol formation, under these conditions, is reduced about 6-fold compared to the 48 h control sample.

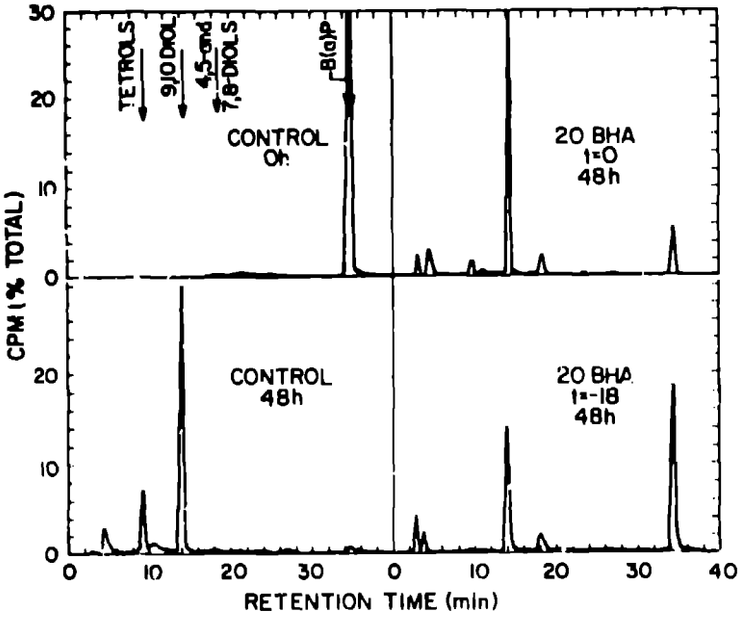

FIGURE 5. Reverse phase HPLC elution patterns of ethyl acetate extracted BaP metabolites from cultures of X-irradiated SHE cells treated with 0 or 20 μg/ml BHA. Arrows indicate elution positions of designated, authentic standards. Data is plotted as the percent total of radioactivity recovered after chromatography.

DISCUSSION

A number of studies indicate that antioxidants can modify the intrinsic properties of metabolic activation systems. However, due to the complexity in these responses, the mechanisms by which these antioxidants behave have been difficult to resolve from *in vivo* studies alone. The present study suggests that cultured SHE cells, capable of metabolizing polycyclic aromatic hydrocarbons, may be useful in investigating these mechanisms *in vitro*. BaP metabolism

in cultured cells has been extensively studied by a number of laboratories (2,14,16) employing organic extraction and HPLC techniques. These investigations provide a solid experimental approach by which the mechanistic action of BHA might be more effectively probed. We have utilized this approach in combination with three biological endpoints (cytotoxicity, mutagenicity, and cell transformation) to monitor the effects of antioxidants such as BHA.

Our data (Table I, Figure 3) indicate that BHA causes a substantial reduction in the mutation frequency at the HGPRT locus induced in CHO cells by BaP. These results are consistent with those reported by Katoh et al. (10) for BHA effects on mutation frequencies induced at the ouabain locus in V79 cells. There was, however, a difference in the concentrations of BHA required to achieve maximum inhibitory effects, i.e., 10 µg/ml reported by Katoh et al. versus 40 µg/ml or greater in this study. These differences can probably be attributed to (a) variations in the responsiveness of the genetic loci being examined (HGPRT vs ouabain) and/or (b) variability in the composition of BHA isomers in the different commercial sources used. In regards to the latter possibility, Wattenberg et al. (24) has shown that the 2-tert-butyl-4-hydroxyanisole isomer is biologically more active than the 3-tert-butyl-4-hydroxyanisole isomer.

The data depicted in both Figure 3 and Table I also indicate that pretreatment of SHE cells with BHA can further enhance its effects on the metabolism and biological properties of BaP. Current efforts in this area are aimed at (a) resolving more precisely the pretreatment timing necessary to maximally affect BaP metabolism and (b) determining whether continued exposure of pretested cells to BHA is necessary for expression of its inhibitory effects.

The reduction in conversion of BaP into water soluble metabolites by increasing BHA concentrations (Fig. 4) suggests either (a) a reduction in overall metabolism of BaP or (b) a reduction in conjugation of BaP metabolites to more water soluble moieties. Our examination of extracellular organic solvent extracts by reverse phase HPLC techniques indicates that the former is more probable, i.e., the amount of unoxidized BaP in reaction mixtures of BHA-treated samples is always higher.

This study demonstrates that SHE cells may be effectively utilized to concurrently measure the effects of antioxidants on carcinogen metabolism and induced mutagenicity and morphological transformation. Our results agree with those current notions which suggest that BHA inhibits the mutagenic and neoplastic properties of certain

procarcinogens by affecting their overall metabolism.

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