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EFFECT OF 2(3)-*TERT*-BUTYL-4-HYDROXYANISOLE ADMINISTRATION ON THE ACTIVITIES OF SEVERAL HEPATIC MICROSOMAL AND CYTOPLASMIC ENZYMES IN MICE*

YOUNG-NAM CHA[1] and ERNEST BUEDING[2]

Departments of Pathobiology[1,2], Environmental Toxicology[1] and Pharmacology and Experimental Therapeutics[2], Johns Hopkins Medical Institutions, Baltimore, MD 21205, U.S.A.

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Abstract—Administration of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) (0.75%) caused a marked increase in the activities of several hepatic enzymes in CD-1 mice. This was associated with increased liver weights and total protein contents, especially of the microsomal and cytosol fractions. While the specific content of cytochrome P-450 was decreased slightly in microsomes, the specific content of cytochrome b_5 and the specific activities of cytochrome *c* reductases (NADPH- or NADH-dependent) were increased (2-fold). In spite of a slight decrease in the specific activities of aminopyrine demethylase and of benzo(a)pyrene hydroxylase, both aniline hydroxylase and UDP-glucronyltransferase activities were increased (2.7- and 4.6-fold respectively). The specific activity of a microsomal membrane marker enzyme, glucose-6-phosphatase, was decreased slightly (−25 per cent). In the cytosol fraction, the specific activities of glucose-6-phosphate dehydrogenase and of UDP-glucose dehydrogenase were increased (3.8- and 6.1-fold respectively). Differences were noted in the time courses of increase and decrease in these enzyme activities after initiation and discontinuation of BHA treatment.

Carcinogenic effects caused by a wide variety of chemicals can be reduced by the administration of certain antioxidants which are used as food additives [1–11]. The most extensive work of this type has been carried out with 2(3)-*tert*-butyl-4-hydroxyanisole (BHA)† 3, 5-di-*tert*-butyl-4-hydroxytoluene (BHT), and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin) by Wattenberg *et al.* [4, 5, 7, 9, 10] and has been confirmed by Weisburger *et al.* [11]. Furthermore, recent studies from our laboratories have shown that the intramuscular administration of benzo(a)pyrene (BP) to mice results in the formation of mutagenic metabolites whose levels could be reduced markedly if the mice injected with this hydrocarbon were fed a diet supplemented with either BHA or ethoxyquin [12].

These observations raise the questions of whether and in what manner these antimutagenic and anticarcinogenic effects can be related to hepatic drug metabolism. Some of the previous studies dealing with these antioxidants have been concerned only with the oxidative aspects of hepatic microsomal drug metabolism and have led to confilicting results [13, 14]. Since drug metabolism *in vivo* is not limited to oxidation but also includes reduction, conjugation and other reactions, the generation and disposition of mutagenic or carcinogenic metabolites may be controlled by many reactions, the majority of which occur in the liver. Thus, the present investigation deals with determination of the activities of hepatic enzymes which might be involved in either the formation or the inactivation of mutagenic metabolites. The study complements others from this laboratory dealing specifically with increased activities of microsomal epoxide hydratase [15] and cytosol glutathione *S*-transferases in the liver [16].

MATERIALS AND METHODS

Female CD-1 mice (Charles River, Wilmington, MA), 6- to 8-weeks-old, were kept in hanging stainless steel wire cages with free access to water and food. Mice were divided into two groups, one of which was fed a powdered diet containing 0.75% BHA (w/w) (Sigma Chemical Co., St. Louis, MO). Based on a normal daily food consumption (2 g), each mouse took 15 mg BHA/day. Unless specified otherwise, these diets were fed for 10 days.

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† The following abbreviations are used in the text in Table 2 and in the figures: BHA: 2(3)-*tert*-butyl-4-hydroxyanisole; BHT: 3,5-di-*tert*-butyl-4-hydroxytoluene; ethoxyquin: 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; BP: benzo(a)pyrene; NADPH-cyto. *c* red.: NADPH-cytochrome *c* reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4); NADH-cyto. *c* red.: NADH-cytochrome *c* reductase [NADH: (cytochrome *c*) oxidoreductase, EC 1.6.99.3]; P-450: cytochrome P-450; b_5: cytochrome b_5; APD; aminopyrine demethylase (aminopyrine, reduced-flavoprotein: oxygen oxidoreductase, EC 1.14.14.1); ANH: aniline hydroxylase (aniline, reduced-flavoprotein: oxygen oxidoreductase, EC 1.14.14.1); BPH: benzo(a)pyrene hydroxylase [benzo(a)pyrene, reduced-flavoprotein: oxygen oxidoreductase, EC 1.14.14.2]; UDPGA Tr: UDP glucuronyltransferase (UDP glucuronate β-glucuronosyltransferase, EC 2.4.1.17); G-6-P'ase: glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9); G-6-PD: glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49); and UDPGD: UDP glucose dehydrogenase (UDP glucose: NAD^+ 6-oxidoreductase, EC 1.1.1.22).

The animals were killed by cervical dislocation and the gross body and liver weights were determined. The livers were homogenized in 19 vol. of 0.25 M sucrose solution using a Teflon–glass homogenizer (six strokes). Microsomes and cytosol fractions were prepared by calcium ion sedimentation from the supernatant fractions of an initial centrifugation of these homogenates for 15 min at 9000 *g* [17]. The microsomal pellet was washed once in 0.25 M sucrose (one-half original homogenate volume) by homogenization and centrifugation, and suspended in 10 ml of the same medium. Aliquots of the microsomal and cytosol fractions were placed into 1-ml capacity vials and frozen in liquid N_2 for storage at −80°. Previous studies with stored samples did not show significant changes in the enzyme activities that have been determined in this study. Protein concentrations were determined by the method of Lowry *et al.* [18] using bovine serum albumin as the standard.

Mixed-function oxidase activities were measured using either aminopyrine (8 mM), aniline (5 mM) or benzo(a)pyrene (100 μM) as substrates according to the methods described previously [19–21]. The contents of cytochromes P-450 and b_5 were determined according to Omura and Sato [22, 23] with a DW-2a spectrophotometer (American Instrument Co., Silver Spring, MD) operating in a split-beam mode. Cytochrome *c* reductase assays (NADPH- or NADH-dependent) were carried out according to Masters *et al.* [24]. UDP-glucuronyltransferase activity was determined with *p*-aminophenol as a substrate according to Mills and Smith [25]. With this substrate, pretreatment of microsomes with detergents for activation was not required. Glucose-6-phosphatase activity was determined according to Swanson [26]. The activities of glucose-6-phosphate dehydrogenase and UDP-glucose dehydrogenase were determined spectrophotometrically according to Löhr and Waller [27].

Various substrates for enzyme assays were obtained from the Sigma Chemical Co. [e.g. cytochrome *c*, glucose-6-phosphate, aminopyrine, aniline, benzo(a)pyrene, etc.] and from Boehringer–Mannheim Biochemicals (Indianapolis, IN) (e.g. NAD^+, NADH, $NADP^+$, NADPH, UDP-glucose, and UDP-glucuronic acid). All other chemicals were of the highest purity available commercially and were not purified further with the exception of aniline and phenol, which were redistilled under nitrogen to yield colorless liquids.

Statistical significance was selected as $P < 0.05$ by Student's *t*-test for paired group analysis.

RESULTS

Dietary administration of BHA for 10 days produced a marked enlargement of the liver, as reported by others [28]. As a result, the relative liver weight (normally 5.7 per cent of bodyweight) was increased to 9.1 per cent. The total amount of liver protein was increased also, though not in proportion to the increase in liver weight. The most marked increases in protein content were found in the isolated microsome and cytosol fraction (Table 1). The specific activities of several enzymes were increased markedly in these fractions (Table 2). Among the microsomal enzymes known to be involved in the oxidation of drugs, there were increases in the specific activities of NADPH-cytochrome *c* reductase and aniline hydroxylase. By contrast the specific activities of aminopyrine demethylase and benzo(a)pyrene hydroxylase were decreased slightly. The specific content of cytochrome P-450 was decreased also in the same proportion as those of aminopyrene demethylase and benzo(a)pyrene hydroxylase activities. UDP-glucuronyltransferase activity, an enzyme involved in the conjugation of hydroxylated metabolites, was increased 4.6-fold. Other microsomal enzyme activities which may or may not be involved in drug metabolism were increased also. For example, NADH-supported cytochrome *c* reductase activities and cytochrome b_5 content were increased 2- to 3-fold. The specific activity of the membrane marker enzyme, glucose-6-phosphatase, was decreased to 80 per cent of control. In the cytosol fraction, the activities of glucose-6-phosphate dehydrogenase and UDP-glucose dehydrogenase were increased 3.8- and 6.1-fold respectively.

The time courses of the BHA-induced increases in specific activities of these enzymes varied considerably (Fig. 1). For example, the specific activity of aniline hydroxylase reached its maximum (2.5 times of control) in 2 days and remained elevated for the entire duration of BHA treatment. Specific activities of other hepatic enzymes reached their maximum levels only after a longer time interval (5–7 days). When BHA was discontinued after 10 days, the activities of these hepatic enzymes returned to control levels, but, again, at different rates. For example, aniline hydroxylase activity returned to its control level within 2 days while up to 7 days were required for the other enzymes.

Table 1. Effects of BHA treatment on size and protein content of livers of mice*

	Control group	BHA-treated group
No. of mice	30	12
Liver weight as a fraction of body weight (%)	5.74 ± 0.35	9.13 ± 0.39
Protein content of homogenate (mg/liver)	111.78 ± 5.99	148.93 ± 8.88
Protein content of microsome fraction (mg/liver)	45.0 ± 2.64	56.2 ± 2.59
Protein content of cytosol fraction (mg/liver)	33.1 ± 1.60	52.36 ± 2.08

*BHA was fed at 0.75% in the diet for 10 days. Other experimental details are given in Material and Methods. The values are given as means ± S. D.

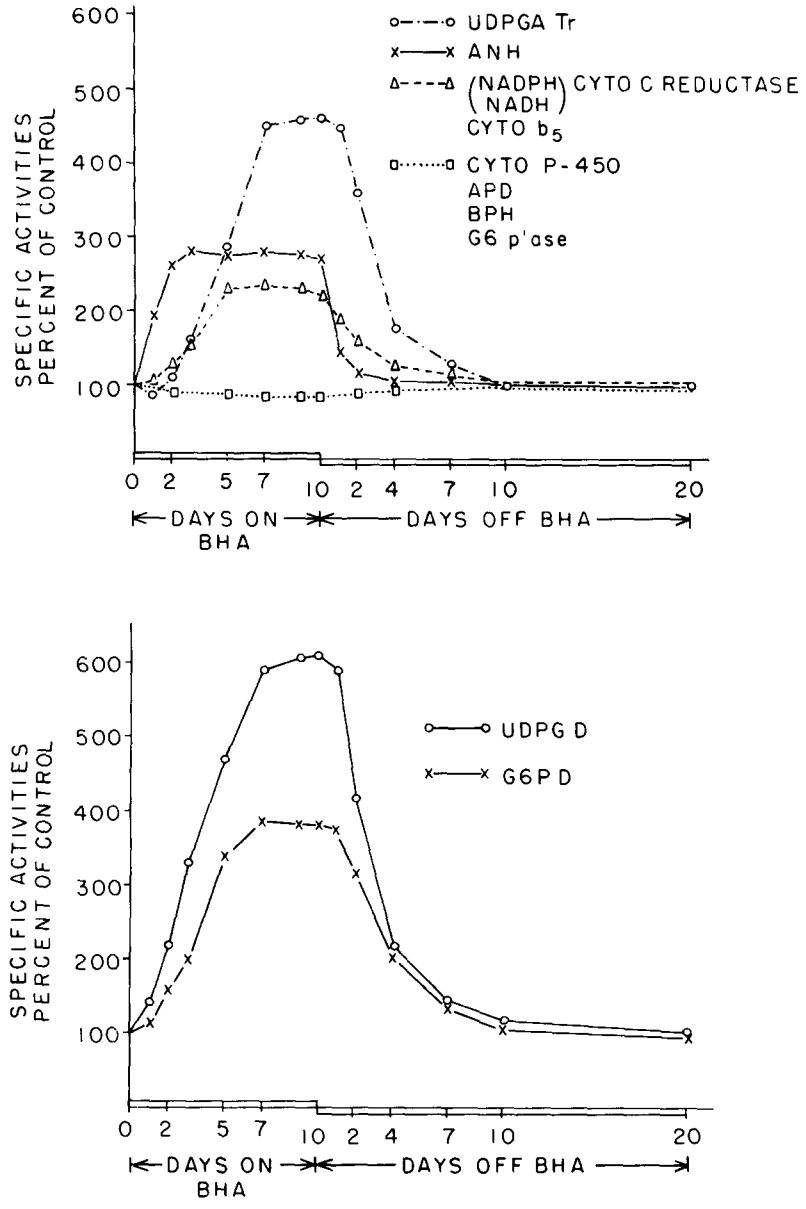

Fig. 1. Effects of initiation and subsequent withdrawal of BHA-containing diet on the time course of specific hepatic enzyme activities. The changes in specific activities of (top) microsomal enzymes: UDP-glucuronyltransferase (UDPGA Tr.), aniline hydroxylase (ANH), NADPH- and NADH-cytochrome *c* reductases, cytochromes b_5 and P-450, aminopyrine demethylase (APD), benzo(a)pyrene hydroxylase (BPH) and glucose-6-phosphatase (G-6-P'ase); and (bottom) cytoplasmic enzymes: UDP-glucose dehydrogenase (UDPGD) and glucose-6-phosphate dehydrogenase (G-6-P-D-), were compared to the control.

DISCUSSION

According to Oesch *et al.* [29], any factors contributing to a decrease in the steady state level of epoxides would reduce the carcinogenic effects of polycyclic aromatic hydrocarbons. Dietary antioxidants might represent such a factor. Their administration was shown to reduce the incidence of tumors induced by a wide variety of chemical carcinogens [1–11]. Furthermore, administration of BHA or ethoxyquin to mice resulted in a drastic reduction in the levels of mutagenic metabolites which could be induced by the injection of benzo(a)pyrene [12]. Although the mechanisms by which these antioxidants inhibit carcinogenic and mutagenic effects are not known, Wattenberg *et al.* [30] have pointed out that this may be caused either by direct interactions between the antioxidant and the ultimate (or proximate) carcinogens, or indirectly by modification of the activities of enzymes catalyzing the metabolism of carcinogens.

Generation as well as the removal of mutagenic or carcinogenic metabolites is controlled by many reactions *in vivo* and the majority of these occur in the liver. In the present and other investigations [15,16], activi-

Table 2. Effects of BHA treatment on hepatice microsomal and cytoplasmic enzyme activities of mice*

	Control group	BHA-treated group
No. of animals	30	12
Microsomal enzymes		
NADPH-cytochrome *c* reductase (μmoles cytochrome *c* reduced/min/mg)	0.712 ± 0.028	1.31 ± 0.07
NADH-cytochrome *c* reductase (μmoles cytochrome *c* reduced/min/mg)	4.03 ± 0.24	8.22 ± 0.35
Cytochrome P-450 (nmoles/mg)	1.53 ± 0.14	1.12 ± 0.10
Cytochrome b_5 (nmoles/mg)	0.656 ± 0.061	1.51 ± 0.07
Glucose-6-phosphatase (μmoles P_i/min/mg)	0.509 ± 0.029	0.413 ± 0.066
Aminopyrine demethylase (nmoles formaldehyde/min/mg)	18.7 ± 1.46	13.9 ± 0.84
Aniline hydroxylase (nmoles *p*-aminophenol/min/mg)	2.25 ± 0.16	6.08 ± 0.59
Benzo(a)pyrene hydroxylase (nmoles 3-hydroxybenzo (a) pyrene/min/mg)	2.07 ± 0.16	1.46 ± 0.19
UDP-glucuronyltransferase (nmoles UDP-*p*-aminophenol/min/mg)	25.2 ± 3.15	117.3 ± 8.3
Cytoplasmic enzymes		
Glucose-6-phosphate dehydrogenase (nmoles NADPH/min/mg)	23.3 ± 5.1	88.2 ± 5.0
UDP-glucose dehydrogenase (nmoles NADH/min/mg)	9.79 ± 1.24	59.8 ± 10.4

*Enzyme specific activities are expressed in the units indicated in parentheses as means ± S. D. BHA was fed at 0.75% (w/w) in the diet for 10 days. Other experimental details are given in Material and Methods.

ties of these hepatic enzymes that might be involved in either the formation or the inactivation of mutagenic metabolites were examined. According to previous reports, BHA treatment did not affect the activities of hepatic microsomal mixed-function oxidases in the rat. However, moderate changes have been observed with other antioxidants, such as BHT [31] and ethoxyquin [32]. As found in the present study, treatment of mice with BHA resulted in a selective increase in the specific activity of aniline hydroxylase (ring hydroxylation). By contrast, the activities of other microsomal mixed-function oxidases were decreased [15], as reflected by the reduced activities of aminopyrine *N*-demethylase and benzo(a)pyrene hydroxylase activities [fluorometric assay of only 3-OH benzo(a)pyrene] or by a lower content of cytochrome P-450. The observed selective increase in the phenolic hydroxylation of aniline could explain the general increase in the levels of phenolic metabolites of benzo(a)pyrene [33, 34]. This could account also for the reduced epoxide levels reported after an *in vitro* incubation of this hydrocarbon with liver microsomes of BHA-treated mice [33, 34]. Similarly, an 11-fold increase in the specific activity of microsomal epoxide hydratase [15], an enzyme catalyzing the hydrolysis of the epoxide ring, could explain anticarcinogenic and antimutagenic effects. They could be brought about also by a shift in the metabolic profile of aromatic metabolites, thereby reducing the steady state levels of epoxides and the carcinogenic effects of polycyclic aromatic hydrocarbons, analogous to the *in vitro* model for the elimination of mutagenic activities of benzo(a)pyrene epoxides postulated by Wood *et al.* [35, 36]. However, an increase of epoxide hydratase activity, under certain circumstances, may contribute to the formation of products with greater carcinogenic and mutagenic properties through the formation of diol-epoxides [37].

The activity of UDP-glucuronyltransferase, a microsomal enzyme catalyzing the conjugation of phenolic metabolites, is increased markedly in the liver of mice treated with BHA. Similarly, a 6-fold increase in the activity of UDP-glucose dehydrogenase in the cytosol could provide more UDP-glucuronic acid to be utilized by the enhanced UDP-glucuronyltransferase activity. Finally, the increased activity of glucose-6-phosphate dehydrogenase, a principal cytosol enzyme involved in the generation of NADPH, could provide more NADPH and lead to the generation of more reduced glutathione. This is consistent with the BHA-induced rise in the levels of acid-soluble sulfhydryl compounds in the liver and other tissues [12]. In this connection, a dose- and time-dependent increase of greater than 10-fold in glutathione *S*-transferases of mouse hepatic cytosol was observed [16].

Because of the multiplicity of enhanced enzymatic activities resulting from treatment with BHA, it is as yet difficult to assign specifically either one or several enzyme activities to the mechanism of antimutagenic and anticarcinogenic effects of this antioxidant. In addi-

tion, the possibility must be taken into account that other, hitherto undetermined, enzymatic reactions may be involved in such a mechanism.

There are differences in the time courses of increase and decrease in the activities of various enzymes after initiation and discontinuation of BHA treatment. Detailed examination of such differences might provide opportunities to correlate more precisely the antimutagenic effects with a given enzymatic activity affected by treatment with an antioxidant

In addition to its anticarcinogenic effect, BHT has also been reported to enhance carcinogenic effects of chemicals [38]. The anticarcinogenic effects were produced by BHT when it was administered before or with carcinogens, while an enhancement of carcinogenic activities was brought about when included in the diet for many months after the carcinogen had been administered. Thus, the elucidation of particular enzymes whose enhanced activities are responsible for the anticarcinogenic and antimutagenic effects might provide opportunities for the design and development of more selective approaches to reduce chemical mutagenesis and carcinogenesis of environmental and therapeutic compounds.

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