Effects of butylated hydroxyanisole on microsomal monooxygenase and drug metabolism

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ABSTRACT Butylated hydroxyanisole (BHA) has been shown previously to inhibit chemical carcinogenesis in experimental animals. When BHA was administered ig or through the food to ♀ mice, the short term effect was the prolongation of hexobarbital sleeping time. Liver microsomal hexobarbital hydroxylase activity was inhibited by BHA added *in vitro*. BHA also inhibited the microsomal metabolism (demethylation) of ethylmorphine, benzphetamine, and *p*-nitroanisole. Long term treatment with dietary BHA, however, induced the microsomal hexobarbital hydroxylase activity and shortened the sleeping time. The induction was accompanied by a decrease of a 43 000 dalton protein species and the intensification of certain proteins in the 49 000-57 000 dalton region. Microsomal demethylase activities with ethylmorphine, benzphetamine and *p*-nitroanisole were not increased by dietary BHA. The results suggest that specific cytochrome P-450 enzymes are induced by BHA pretreatment.

KEY WORDS butylated hydroxyanisole; drug metabolism; cytochrome P-450; hexobarbital; microsomes; monooxygenases; *O*-demethylating oxidoreductases

Butylated hydroxyanisole [2(3)*tert*-butyl-4-methoxyphenol] (BHA) is a free radical quencher used commonly as a food-additive. Commercial BHA is a mixture of 2- and 3-*tert*-butyl hydroxyanisole isomers, 15% and 85% respectively[1]. Toxicity is seen only at extremely high doses, with LD_{50} higher than 2 g/kg for most animal species[1,2]. When added to the diet at a level of 5 mg/g diet, BHA reduced the incidence of tumorigenesis in animals induced by a variety of chemicals[3]. Dietary BHA induced the activities of glutathion *S*-transferase, epoxide hydrolase, UDP-glucuronyltransferase, glucose-6-phosphate dehydrogenase, and NADPH-quinone reductase in mice[4-6]. Some of these enzymes are phase II enzymes involved in the detoxification and excretion of xenobiotics.

BHA affected the cytochrome P-450 (P-450) dependent monooxygenase system. BHA affected microsomal metabolism of benzo pyrene and decreased the covalent binding of benzo pyrene metabolites to DNA[7-10]. The *in vivo* effects of BHA consumption, however, are not known. In the present report, we describe the effects of BHA treatment on drug metabolism *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals BHA, isocitrate dehydrogenase, DL-isocitric acid, NADP, and NADPH were obtained from Sigma Chemical Co, *p*-nitroanisole was from Eastman Organic Co. Chemicals received as gifts were: ethylmorphine-HCl from Merck & Co, benzphetamine-HCl from Upjohn Co, and sodium hexobarbital from Winthrop Lab.

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Treatment of mice and determination of hexobarbital sleeping time Female Swiss Webster mice (♀, 18-20g) were obtained from Taconic Farms (Germantown NY) and were fed "Wayne lab-Blox" (Allied Mills). In the dietary BHA experiments, one group of mice received a 0.5% BHA supplement in the diet for 7 d and the control group received no supplement. In the short term BHA experiments, the mice received ig different doses of BHA in 0.1 ml olive oil, 0.5-4 h before the hexobarbital treatment; the control group received only the vehicle. Sodium hexobarbital was injected ip in saline at 100 mg/kg. Sleeping time was measured as the time between disappearance and restoration of the righting reflex.

Microsomal preparation and enzyme assays The mice were decapitated. Liver microsomes were isolated by differential centrifugation and stored at −86℃[11]. Protein, P-450, and NADPH-cytochrome C reductase were determined by established methods[11]. The hexobarbital hydroxylase activity was determined by the disappearance of the substrate[12].

Gel electrophoresis Gel electrophoresis was performed with a discontinuous slab gel system[11].

RESULTS

Effects of BHA on hexobarbital sleeping time When BHA was given ig 0.2 g/kg 4 or 0.5 h before hexobarbital, the hexobarbital sleeping time was prolonged to 298% and 357% of the control group, respectively (Tab 1). A similar effect was produced by maintaining the mice on a 0.5% BHA fortified diet for 18 h. A longer period of feeding with BHA shortened hexobarbital sleeping time. After the mice were maintained on the 0.5% BHA diet for 2, 3, or 10 d, the hexobarbital sleeping time of the mice was shortened to 60% that of the control. No significant difference was seen among the groups which had been treated for 2, 3, or 10 d. Different doses of BHA were given ig 1 h before hexobarbital. A dose-response curve was obtained (Fig 1). The sleeping time doubled at a BHA dosage of 25 mg/kg ($p<0.01$) and increased 5-fold with 200 mg/kg

Tab 1. **Effects of BHA on hexobarbital sleeping time in mice.** $\bar{x}\pm SD$. $^{**}p<0.05$, $^{***}p<0.01$

Treatment	n	Sleeping time (min)	%
Control	10	42±26	100
BHA, 0.5 h	10	150±46***	357
BHA, 4.0 h	10	125±34***	298
Control diet	15	28±10	
BHA diet, 18 h	15	42±24**	150
Control diet	20	42±20	
BHA diet, 2 d	20	27±14**	64
Control diet	10	51±17	
BHA diet, 3 d	10	30±17***	59
Control diet	25	35±15	
BHA diet, 10 d	24	21±10***	60

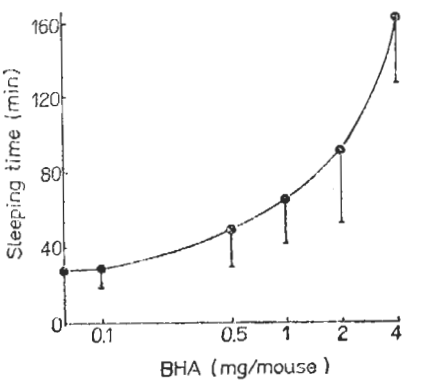

Fig 1. **Prolongation of sleeping time by ig BHA 1h before sodium hexobarbital. 20 mice/group.** $\bar{x}\pm SD$

Induction and inhibition of hexobarbital hydroxylase by BHA Tab 2 showed that treatment with 0.5% BHA diet for 3 d increased the NADPH-dependent hexobarbital hydroxylase activity by 44%, though it did not change the gross microsomal

P-450 content and appeared to increase the NADPH-cytochrome *c* reductase activity only slightly. BHA 100 μ*M* caused 65% and 50% inhibition of the hydroxylase activity of the control and BHA-treated mice, respectively.

Effects of dietary BHA treatment on the pattern of microsomal proteins Polyacrylamide gel electrophoresis of microsomal samples revealed alteration of protein band pattern by the dietary BHA treatment (Fig 2). Most notable is the disappearance of a 43 000 dalton band. The treatment also intensified a protein band of 49 000 daltons possibly epoxide hydrolase which is known to be induced by BHA[13]. The epoxide hydrolase activity, measured with benzo pyrene-4,5-oxide as the substrate[14], was increased 6 to 7-fold by the BHA diet from an average activity of 1.26 nmol/min/mg for control microsomes. Also evident was the change in the band pattern in the 50 000 to 57 000 region, especially the intensification of the bands at 51 000 and 55 000 daltons in the BHA-induced microsomes. We believe that some of the induced protein bands contain P-450 isozymes.

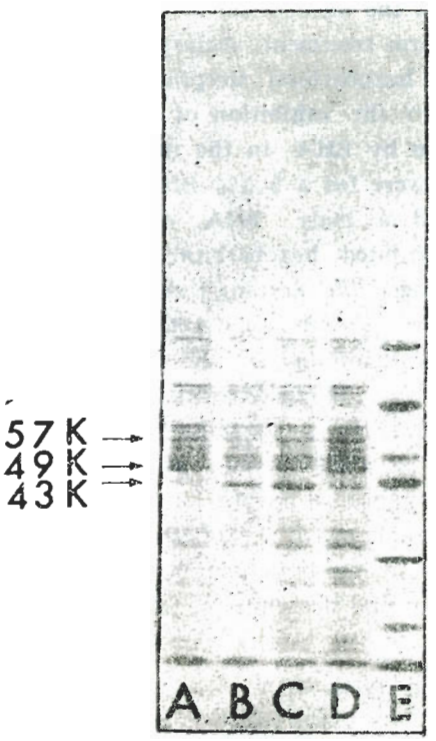

Fig 2. Gel electrophoresis of microsomal proteins. Each well contained 5.3 μg protein of the following samples; A) Dietary BHA-induced microsomes; B) Control microsomes; C&D) Control and dietary BHA-induced microsomes, respectively, from a different experiment; E) 7 protein standards from bottom to top are α-lactalbumin (mol wt 14 000, at the buffer front), trypsin inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), P-450 (52 000), albumin (67 000), phosphorylase b (94 000).

Effects of BHA on the oxygenation of other substrates In contrast to microsomal hexobarbital hydroxylase activity, the ethylmorphine, benzphetamine, and *p*-nitroanisole demethylase activities were not increased by the dietary BHA treatment (Fig 3). The ethylmorphine demethylase was actually decreased by the treatment. When added to the incubation mixture, BHA was an inhibitor of all the 3 activities assyed (Fig 3). A 50% inhibition of

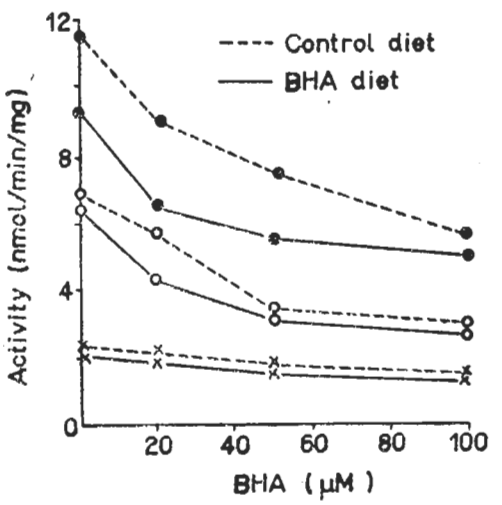

Fig 3. Inhibition of drug metabolism by BHA. The assay mixture contained liver microsomes. (0.8 mg protein) from BHA-fed (2 wk) mice or control mice and 5 m*M* ethylmorphine (●), 1 m*M* benzphetamine (○), or 1 m*M* *p*-nitroanisole (×). BHA was added in 10 μl acetone.

ethylmorphine and benzphetamine demethylase activities was seen with *c* 100 μ*M* of BHA and the *p*-nitroanisole demethylase appeared to be less susceptible to the inhibition. No difference in the susceptibility to BHA inhibition was observed between BHA-induced and control microsomes.

Because ethylmorphine demethylase activity was higher than other monooxygenase activities, it was selected for investigating the mechanism of inhibition by BHA (Fig 4). The presence of BHA increased the K_m from 1.63-2.12 m*M* decreased the V_{max} of reaction from 20.9-13.7 nmol/min/mg. The result indicates a mixed inhibition consisting of both competitive and non-competitive modes of inhibition.

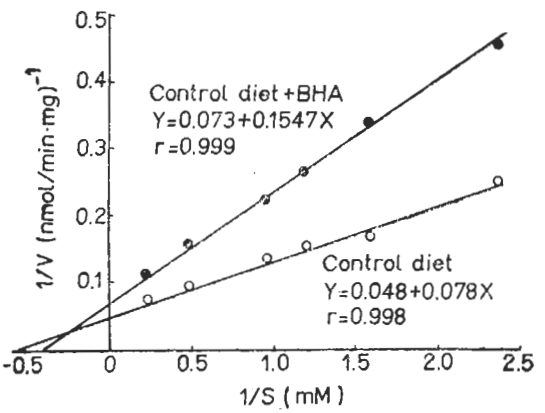

Fig 4. Double reciprocal plots of ethylmorphine demethylase reaction effect of BHA. The incubation mixture contained control microsomes (0.8 mg protein) and ethylmorphine at the concentrattion indicated in the presencc (●) or absence (○) of 50 μ*M* BHA.

DISCUSSION

Dietary BHA treatment altered the metabolism of drugs and carcinogens when assayed *in vitro*[3-9]. In the present work, dietary BHA treatment shortened hexobarbital sleeping time in mice. This effect is attributable to the induction of hexobarbital hydroxylase (Tab 2), a rate-limiting enzyme in the metabolism of this drug. In a short term treatment, dietary or ig BHA prolonged hexobarbital sleeping time due possibly to the inhibition of hexobarbital metabolism by BHA in the tissues. When the mice were fed a 0.5% BHA diet, they maintained a body BHA concentration which inhibited hexobarbital metabolism and prolonged the sleeping time by approximately 50% (Tab 1). After the mice have been maintained on the BHA diet for more than 2 d, the hexobarbital metabolism was affected by 2 contradictory factors. The cellular BHA should still have an inhibitory effect on hexobarbital metabolism but the induction of hexobarbital hydroxylase appeared to be a dominant factor and the hexobarbital sleeping time was shortened.

Tab 2. Induction and inhibition of hexobarbital hydroxylase activity by BHA.

BHA diet 0.5% 3 d	P-450 (nmol/mg)	Reductase[1] (unit/mg)	Hexobarbital hydroxylase activity, (nmol/min/mg)	
			no BHA[2]	with 100 μ*M* BHA[3]
−	1.10	167	1.83±0.37	0.61, 0.66
+	1.12	224	2.63±0.43	1.71, 0.94

[1] NADPH-cytochrome *c* reductase assayed at 25℃. One unit of activity corresponds to the reduction of 1 nmol cytochrome *c*/min.

[2] $\bar{x}$±SD of 4 determinations with 20 mice for each group; the treated group is significantly different ($p<0.05$) from the control.

[3] 2 determinations with 20 mice.

Although pretreatment with BHA enhanced hexobarbital hydroxylase activity, it did not increase the demethylase activities of ethylmorphine, benzphetamine, and *p*-nitroanisole. It is possible that BHA treatment selectively induces certain forms of P-450 isozymes which are more efficient in the metabolism of hexobarbital but not other types of substrates such as ethylmorphine. Consistent with this are the BHA-induced changes in the protein band pattern in the 50 000-57 000 dalton region (Fig 2). This concept is supported by our previous observation that BHA pretreatment altered the region selectivity in the metabolism of benzopyrene by mouse liver microsomes and decreased the K_m and increased the V_{max} values of *O*-ethoxycoumarin dealkylase[7].

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丁羟茴醚对微粒体单加氧酶及药物代谢的作用

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提要 小鼠ig丁羟茴醚(BHA) 0.5,4 h或喂以含0.5% BHA食料18 h后，海索巴比妥钠睡眠时间延长为对照组的150-357%。BHA食料摄入2，3及10 d后，睡眠时间缩短为59-64%，肝微粒体海索巴比妥羟化酶活性增加。这种诱导作用伴有微粒体蛋白电泳图谱在分子量49 000-57 000间某些蛋白区带的深染。提示BHA可以诱导一种特殊的P-450酶。

关键词 丁羟茴醚；药物代谢；细胞色素P-450；海索比妥；微粒体；单加氧酶；*O*-去甲基氧化还原酶

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