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Effect of p-Dimethylaminoazobenzene and 2(3)-*tert*-Butyl-4-hydroxyanisole on Lipid Peroxidation, Glutathione-S-transferase, Peroxidase and Reductase in Rat Liver

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An experiment was conducted in order to investigate the effect of p-dimethylaminoazobenzene (DAB) and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) on the lipid peroxidation and peroxide-destroying enzyme system in the rat liver. Dietary supplementation of DAB (0.06%) for three weeks caused the elevation of glutathione-S-transferase activity by 60% and glutathione reductase by 50%, but it decreased glutathione peroxidase and catalase activities significantly. Dietary supplementation of BHA (0.75%) also increased glutathione-S-transferase activity in the liver by 2 folds, and it counteracts DAB effect on the glutathione peroxidase and catalase activities. There was a marked increase in malondialdehyde content in the postnuclear fraction of liver by the treatment of DAB, but the addition of BHA lowered the malondialdehyde content to almost the control level. The protective effect of BHA on the lipid peroxidation induced by DAB administration at the enzyme level seems to be due to the induction of glutathione-S-transferase and the protection of glutathione peroxidase and catalase activities from being lowered by DAB administration.

Key Words: DAB, BHA and lipid peroxidation.

The reactive forms of chemical carcinogens are electrophilic derivatives which are formed during the metabolism of procarcinogens in the host tissues (Miller and Miller, 1977). Labuc and Blunck (1979) demonstrated that 3'-methyldimethylaminoazobenzene is metabolized to at least two electrophilic metabolites in the rat liver cell-free system which required NADPH/NADH generating system and the addition of reduced glutathione to the system inhibited the covalent binding of these metabolites to RNA and tissue protein. The conjugation of electrophilic derivatives with reduced glutathione is catalyzed by a soluble enzyme, glutathione-S-transferase. This enzyme has a variety of electrophilic, hydrophilic substrates including organic peroxides. In addition to its catalytic role, the enzyme binds a number of ligands that are not substrate (Habig *et al.*, 1974). Glutathione-S-transferase is widely distributed in various animal tissues and is inducible by a variety of chemicals such as phenobarbital, 3,4-benzo(α)pyrene and 3-methylcholanthrene (Clifton and Kaplowitz, 1978).

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Previously, we have observed that 3′-methyl-4-dimethylamino-azobenzene as well as phenobabital treatment increased glutathione-S-transferase activity in the liver of mice (Lee and Oh).

Recently, lipid peroxidation and its products such as malondialdehyde are considered as a cause of carcinogenesis (Shamberger *et al.*, 1974). The suppressive effect of antioxidants on tumor production in a variety of rodent tissues by chemical carcinogen was reported (Wattenbert LW, 1978). Although the role of antioxidants in the suppression of carcinogenecity has not been fully understood, they may act as scavenger for free radicals which are considered as ultimate carcinogens. BHA is one of classical antioxidants widely used as food additives and it showed no toxicity in animals (Branen, 1975). Since the liver is one of the most important organs for the metabolism of various chemicals and is responsible for the major detoxification, the induction of glutathione-S-transferase in the liver will have a significance in the metabolism of organic peroxides generated. It has been reported that the specific activity of glutathione-S-transferase was drastically increased in the liver of mice fed a diet supplemented with 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) with no changes in hepatic mixed-function oxidase activities (Batzinger *et al.*, 1978). Another enzyme which can decompose the organic peroxide is glutathione peroxidase. This enzyme also destroys the hydrogen peroxide as catalase does. A common substrate for the glutathione-S-transferase and glutathione peroxidase is reduced glutathione, which is regenerated by NADPH via glutathione reductase. The present study was undertaken to investigate the effect of DAB and BHA administrations on the lipid peroxidation, glutathione-S-transferase, glutathione peroxidase, glutathione reductase and catalse activities.

MATERIALS AND METHODS

Chemicals

p-Dimethylaminoazobenzene was purchased from Eastman Kodak Co. (Rochester, N.Y., USA). 2(3)-*tert*-Butyl-4-hydroxyanisole, glutathione, yeast glutathione reductase and NADPH were supplied by Sigma Chem. Co.. St. Louis, USA). 1-Chloro-2,4-dinitrobenzene was obtained from Merk (Darmstadt, W. Germany).

Treatment of Animals

Forty eight male albino rats (150-250 g) were divided into 3 groups. Another 4 rats were the control group. All rats were fed with a basal diet for one week and each group of animals (16 rats) received their respective diets which were prepared by supplementation of BHA (0.75%), DAB (0.06%) and BHA plus DAB (0.75% BHA + 0.06% DAB) to the basal diet. Control rats received only basal diet for 4 weeks. Basal diet contained 660 gm of glucose, 180 gm of milk casein, 40 gm of salt mixture (Nutritional Biochemical Corp., Ohio, USA), 100 gm of corn oil, 20 gm of cod liver oil, 1.5 gm of choline chloride, 50 mg of Vit. K_3, 20 mg of riboflavin, 20 mg of thiamine, 20 mg of pyridoxine, 60 mg of calcium pantothenate, 50 mg of nicotinamide, 1.8 mg of folic acid, 0.6 mg of biotin, 100 mg of inositol, 50 mg of p-aminobenzoate, 40 μg of cyanocobalamine per kg of diet. 4 rats in each group were sacrificed at 3, 7, 14 and 21 days after treatment. The liver from each animal was rapidly removed, weighed and chilled. Five grams of each liver was sampled and homogenized with a teflon pestle tissue homogenizer in three volume of cold TKM buffer (0.25 M Tris-HCl, pH 7.4, containing 2.5 mM KCl and 5 mM $MgCl_2$). The homogenate was centrifuged at 600 g for 10 min and the pellet designated as

the nuclear fraction was removed. The supernatant fraction was used for the thiobarbiturate test (TBA test). The postnuclear fraction was further centrifuged at 105,000 g for 60 min and the supernate was designated as soluble fraction and was used for enzyme assays.

Measurement of malondialdehyde contents

Malondialdehyde content in the postnuclear fraction was measured by the method described by Ernster and Nordenbrand (1967). The reaction mixture containing 2 ml of 30% trichloroacetic acid, 0.2 ml of 5 M HCl, 2 ml of 0.75% thiobarbituric acid and 2 ml of postnuclear fraction was put in a boiling water bath for 15 min. The reaction mixture was centrifuged at 2,000 g for 15 min and the supernate was decanted to measure the absorbance at 535 nm. An extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used for the calculation of malondialdehyde content.

Enzyme assays

Glutathione-S-transferase activities were measured by the method of Habig (1974). The reaction mixture (1 ml) contained 0.1 M potassium phosphate buffer (pH 6.8), 1 mM 1-chloro-2,4-dinitrobenzene, 1 mM reduced glutathione. The changes in absorbance at 340 nm were recorded and the activities were calculated using the millimolar extinction coefficient of 9.6. Glutathione reductase activities were assayed by the method of Racker (1955). The reaction mixture (1 ml) contained 60 mM potassium phosphate buffer (pH 7.6), 0.1 mM NADPH, 0.1% bovine serum albumin and 2 mg of oxidized glutathione. Glutathione peroxidase activities were measured by the modified method of Paglia and Valentine (1967). The reaction mixture contained 80 mM potassium phosphate buffer (pH 7.0), 1 mM sodium azide and 1 unit of yeast glutathione reductase, 3 mM EDTA, 2 mM reduced glutathione, 0.1 mM NADPH, 0.12 mM H_2O_2. The changes in absorbance at 340 nm was recorded and the enzyme activity was expressed as μmole of NADPH oxidized per min. Catalase activities were determined by the method described by Bergmeyer *et al.* (1974). Reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 6.8), 15 mM H_2O_2. Absorbance at 240 nm was recorded and the activity was calculated using the millimolar extinction coefficient of 0.04. Absorbance changes were recorded with Gilford spectrophotometer, Model 240. Protein content was measured by Lowry method (1951) using bovine serum albumin as a standard.

RESULTS

There were increases of the ratios of liver weight to body weight within 3 days for the BHA-treated group and in 2 weeks for the DAB-treated-group. These ratios in the control, the BHA-treated, and the DAB-treated group after 3 weeks were 0.028 ± 0.002, 0.038 ± 0.005, 0.035 ± 0.003, respectively. Feeding of DAB caused a marked elevation of malondialdehyde content in the postnuclear fraction of the liver and BHA supplementation had a reducing effect on it (Fig. 1). There was a significant reduction of malondialdehyde content in the liver of DAB plus BHA-treated group as compared with the DAB-treated group. Glutathione-S-transferase activities in the soluble fractions of the liver of rats fed a diet containing DAB or BHA were significantly higher than that of the control (Fig. 2). Glutathione-S-transferase activity in the liver of rats fed a diet supplemented with both DAB and BHA was also significantly higher than the control level, but the value of the elevaelevation was not additive. Glutathione peroxidase activity in the DAB-treated group was significantly lower than that of the control,

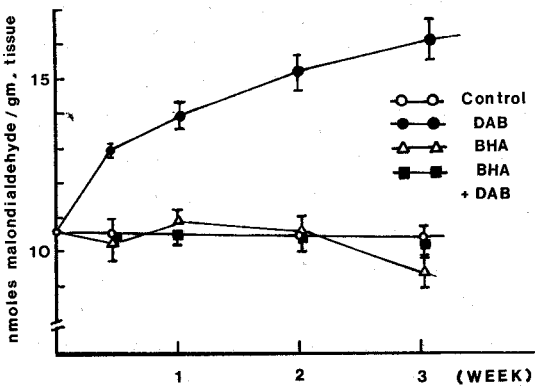

Fig. 1. Effect of DAB and BHA on the malondialdehyde content in the postnuclear fraction of rat liver.

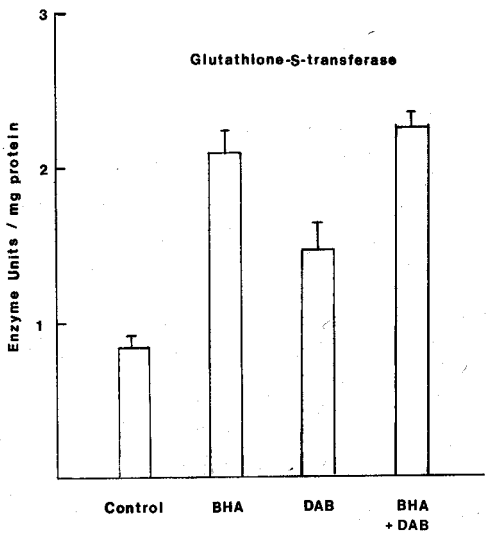

Fig. 2. Effect of DAB and BHA on the glutathione-S-transferase activity in the liver soluble fraction of rats.

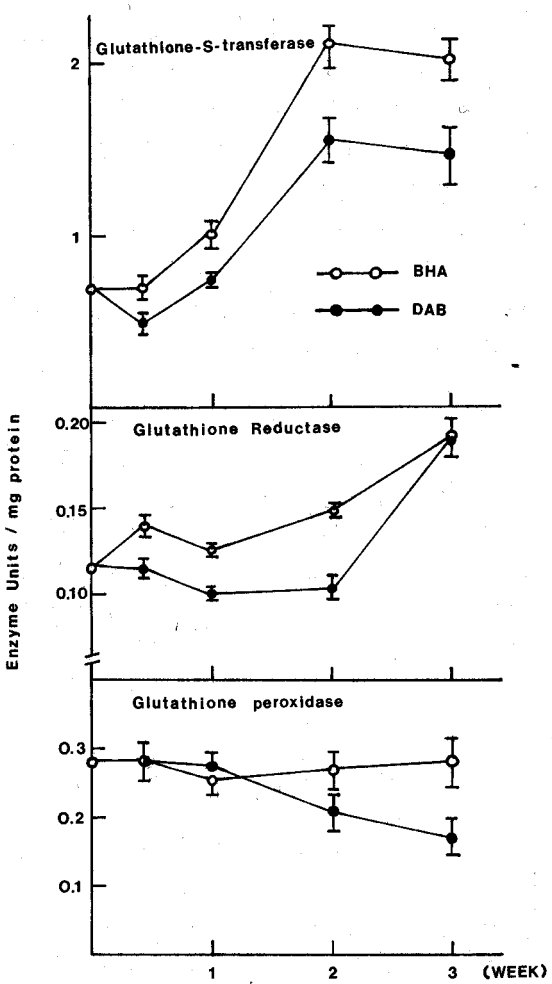

Fig. 3. Changes in glutathione-S-transferase, glutathione reductase and glutathione peroxidase activities by DAB and BHA supplementation.

but no differences in the enzyme activities between the control and DAB plus BHA-treated groups were observed (Table 1). Glutathione reductase activities in DAB, BHA, DAB plus BHA-treated groups were much higher than that of the control, but no significant differences in the activities among the three groups were observed (Table 1). Catalase activity in the DAB-treated group was reduced to an half of the control value and the supplementation of BHA could improve the activity significantly (Table 2). There were gradual increases in glutathione-S-transferase and glutathione reductase activities by DAB and BHA treatment during the period of 3 weeks (Fig. 3). However, glutathione peroxidase activity gradually decreased by DAB treatment with small changes in the activity by BHA treatment.

Table 1. Effect of DAB and BHA on glutathione peroxidase, glutathione reductase and catalase activities in rat liver[a]

Treatment[b]	Glutathione peroxidase	Glutathione reductase	Catalase
Control	0.272±0.029	0.115±0.003	425.0±27.0
BHA	0.283±0.044	0.192±0.005**	377.4±32.7*
DAB	0.170±0.023**	0.190±0.011**	227.9±36.0**
BHA + DAB	0.266±0.025	0.192±0.005**	354.6±37.5*

a. All values are specific activities of enzyme (μmoles of substrate transformed/min/mg protein) ± S.D.
* Significantly different from the control ($P<0.05$)
** Significantly different from the control ($P<0.01$)
b Rats were fed a diet supplemented with each chemical for 3 weeks.

DISCUSSION

DAB, a powerful liver carcinogen, appears to be metabolically activated to ultimate carcinogen probably being electrophile in the liver. And lipid peroxidation is implicated in the genesis of various tumors (Mukai and Goldstein, 1976). We have observed the increase of malondialdehyde in the liver of rats given DAB in this experiment, which indicates the induction of lipid peroxidation by DAB. A decreased malondialdehyde content in the liver of rats fed a diet containing DAB and BHA explains the antioxidant effect of BHA on the DAB—induced lipid peroxidation. The increased glutathione-S-transferase activity observed in the DAB—treated group in the present experiment may be due to the induction of enzyme by DAB metabolites and/or lipid peroxidation products. The increased activity of glutathione-S-transferase in the liver of rats fed with BHA in this study confirms the previous reports that BHA induces this enzyme in rat liver (Benson *et al.*, 1979; Batzinger *et al.*, 1978). The induction of glutathione-S-transferase by dietary BHA may alleviate the toxic effect of electrophilic metabolites including organic peroxide generated by azo-dye feeding *in vivo*. In consideration of the increase of relative liver weight within 3 day by BHA feeding, the total activity of this enzyme per liver is much higher than that of the DAB-treated group (data not included). The effect of the elevation of the enzyme activity by BHA and DAB was not additive when these chemicals were fed together, which indicates the presence of the counteracting effect of BHA against DAB during the metabolism. From these results, it is conceivable that BHA may reduce the toxicity caused by DAB through the glutathione-S-transferase-mediated detoxification system. Glutathione peroxidase and catalase are responsible for the destruction of hydrogen peroxide, and lipid peroxide is thought to be destroyed by glutathione peroxidase glutathione-S-transferase (Little and O'Brien, 1968; Prohaska and Ganther, 1977). Decreased activities of glutathione peroxidase and catalase in the soluble fraction of rats fed a diet containing DAB implicate that DAB decelerates the catabolism of peroxides which may lead to cellular damage. When BHA was added to the diet containing DAB, glutathione peroxidase activity returned to the control level in the present study. The role of BHA in the recovery of glutathione peroxidase seems to protect the enzyme from the damage caused by DAB treatment, since glutathione peroxidase, a selenoenzyme, has been shown to be labile in the absence of a stabilizer (Oh *et al.*, 1974; Nakamura *et al.*, 1974). The increased activity of glutathione reductase by BHA, DAB or both in the present study can lead to an acceleration of the generation of reduced glutathione which is a substrate for both glutathione-S-transferase and glutathione peroxidase although the mechanism for the increment is not known. Taniguchi *et al.* (1974) observed that the ratio of glutathione

peroxidase activity to glutathione reductase activity was increased for the first 1-3 days of DAB feeding, then decreased until 70 days. The results well agreed with our present experimental results. The protective effect of BHA on the lipid peroxidation induced by DAB in rat liver may be explained in part through the induction of glutathione-S-transferase and the prevention of glutathione peroxidase and catalase from being decreased.

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