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Nephrotoxicity of butylated hydroxytoluene in phenobarbital-pretreated male rats

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Abstract. A single large dose of butylated hydroxytoluene (BHT, 1000 mg/kg) in male Fischer 344 rats produced some renal damage, reduced accumulation of *p*-aminohippuric acid in renal slices, proteinuria and enzymuria, in addition to hepatic damage. Further, prior administration of phenobarbital (80 mg/kg, i.p., daily for 4 days) in the high-dose BHT-treated male rats produced renal damage accompanied by slight tubular necrosis. The renal damage was confirmed by biochemical and histological changes. These changes were dose dependent, with a maximum at 24 h after BHT administration, but had returned to the normal range by 48 h. Female rats, on the other hand, were less susceptible to BHT-induced renal and hepatic damage than male rats. The results indicate sex differences in BHT-induced renal or hepatic damage.

Key words: Butylated hydroxytoluene – Nephrotoxicity – Phenobarbital – Rat

Introduction

It is well known that BHT (3,5-di-tert-butyl-4-hydroxytoluene) is widely used as a phenolic antioxidant in processed foods, cosmetics and petroleum products. The acute toxicity of BHT is low. The LD_{50} in the rat is greater than 2 g/kg body weight (Karplyuk 1960). Although BHT is generally considered to be safe at the concentrations present in foods (the acceptable daily intake of BHT for man is 0.125 mg/kg) (WHO Technical Report Ser. 1987), we have demonstrated that high doses of BHT in rats cause hepatic injury accompanied by centrilobular necrosis (Nakagawa et al. 1984, 1985). This finding was recently confirmed in other studies using male Wistar rats (Powell et al. 1986). Further, pretreatment with phenobarbital (PB) in mice treated with BHT plus buthionine sulfoximine produced an increase in serum transaminase (maker enzyme of liver damage) relative to that observed when PB pretreatment was omitted (Mizutani et al. 1987). Previous studies have demonstrated that BHT is converted to a highly reactive intermediate (BHT-quinone methide) by a cytochrome P-450-linked monooxygenase system, and that this metabolite specifically binds to sulfhydryl groups of protein, glutathione and cysteine (Nakagawa et al. 1981, 1983). It has been suggested that BHT-induced hepatotoxicity is related to the formation of active intermediate and to the depletion of hepatic glutathione (Nakagawa et al. 1984). BHT can also induce some renal damage, evidenced by a reduction in the renal transport of organic ions, an indicator of renal function (Ford et al. 1980), and induces histological changes in the renal distal tubule in short-term toxicological studies (Meyer et al. 1978). Since there is little information available on the effects of BHT on renal function, even though a major route of elimination is via the urine (Daniel and Gage 1965; Ladomery et al. 1967; Daniel et al. 1968), the objective of this study was to investigate BHT nephrotoxicity in rats.

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Materials and methods

Materials. The chemical compounds used were obtained from the following companies: BHT and *p*-aminohippuric acid (PAH) from the Wako Chemical Co. (Osaka, Japan), and 3,5-di-tert-butyl-4-hydroxybenzoic acid (BHT-acid) from the Aldrich Chemical Co. Inc. (Milwaukee, Wis, USA); all other chemicals were of the highest purity available.

Animals and treatment. Fischer 344 rats (F344/DuCrj; Charles River Japan Inc. Astsugi, Japan), 8-week-old females (weighing 140–160 g) and males (weighing 180–200 g), were divided into groups of five rats for each experiment, housed in a temperature (20–24° C)- and light (12 h)-controlled room and allowed both food and water ad lib. To evaluate the effects of phenobarbital (PB), an inducer of monooxygenase enzymes, on the metabolism and nephrotoxicity or hepatotoxicity of BHT, rats were pretreated as follows and then dosed orally with BHT (500 or 1,000 mg/kg, in corn oil). PB (80 mg/kg, in saline) was injected intraperitoneally once daily for 4 days prior to BHT administration; the last dose of PB was injected 24 h prior to BHT dosing. The corresponding control animals received physiological saline and corn oil. After treatment with BHT the animals were killed by decapitation at defined intervals and the livers and kidneys removed and weighed.

The blood was allowed to clot for 30 min at room temperature and then centrifuged. The serum was collected for measurement of urea nitrogen (UN) and glutamate-pyruvate transminase (GPT).

p-Aminohippuric acid (PAH) accumulation into kidney slices. After removal, the kidneys were immediately placed

in ice-cold saline. Accumulation of PAH was measured by a modification of the technique of Cross and Taggart (1950), as described previously. Thin slices (approximately 100 mg) of the renal cortex were prepared free-hand and incubated in medium containing 7.4×10^{-5} M PAH. The slices were incubated at 25° C under 100% oxygen for 90 min. Samples were subsequently treated as described by Newton et al. (1982). PAH was determined by the colorimetric method of Smith et al. (1945). The accumulation of PAH in renal slices was expressed as a slice-to-medium (S/M) concentration ratio, where S represents mg PAH per g tissue and M represents mg PAH per ml medium.

Collection of urine and urinalyses. Rats were placed in individual glass metabolic cages (Metabolica, Sugiyama-gen Co., Tokyo, Japan) with free access to food and water. After a 3-day period of acclimation to the cages, the animals were administered BHT. Urinalyses were conducted on individual 24-h urine specimens collected on ice. This was done for 4 consecutive days. At the end of each 24 h period, the volume of each sample was recorded and the protein content was determined by the Bradford Coomassie blue dyebinding method (Bradford 1976) using commercial reagents (Bio-Rad Laboratories, Richmond, CA, USA). Bovine serum albumin was used as the standard. An aliquot of each urine specimen was subjected to Sephadex G-25 column chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove interfering substances and eluted with 0.9% saline. This eluent was used to determine the activities of alkaline phosphatase (AP), lactate dehydrogenase (LDH), and N-acetyl-β-D-glucosaminidase (NAG).

Other analytical methods. The activities of serum GPT, urinary AP, LDH and NAG were determined by UV or colorimetric assay (using a Hitachi 705 automatic analyzer) by the following methods: GPT (Karmen 1955), AP (Kind and King 1954), LDH (Wroblewski and La Due 1955) and NAG (Horak et al. 1981). Each urinary enzyme activity is expressed as nanomoles or micromoles per min per 24 h urine. Serum UN was determined by the urease-indophenol method (Fawcett and Scott 1960).

Histology. Liver or kidney sections were fixed in buffered formalin solution, and paraffin sections were prepared and stained with hematoxylin and eosin.

Statistics. The mean values for each treatment group were compared to the appropriate controls using the multiple *t* statistic of Dunnett. Data of GPT assay were analysed by the Mann-Whitney *U* test.

Results

To determine whether renal damage is induced by BHT in addition to hepatotoxicity, changes in levels of serum UN and GPT activity and in liver and kidney weights were measured in PB-pretreated and untreated rats 24 h after oral administration of BHT. As shown in Table 1, a high dose of BHT (1,000 mg/kg) induced marked elevations in serum UN (about 3.5-fold over control levels) accompanied by a slight increase in relative kidney weight in the PB-pretreated male rats. Hepatic damage induced by BHT was reproduced in this experiment (Fig. 3C). Elevation of serum GPT activity and increase in liver weight were dependent on the dose of BHT in the PB-pretreated rats. In the PB-pretreated rats, the elevation of GPT activity in male rats was about 2-fold greater than that in female rats at either low- or high-doses of BHT. The results in Table 1 indicate that cotreatment of BHT with PB is the cause of renal damage in male rats, and that the hepatic damage induced by BHT is enhanced by PB pretreatment in both female and male rats.

Since Ford et al. (1980) reported that BHT reduced the renal transport of organic acids (PAH), and did not affect renal transport of organic ions, the accumulation of PAH was measured as a parameter of renal cortex function in this experiment. A high dose of BHT in vivo in rats pro-

Table 1. Effect of phenobarbital-pretreatment on BHT toxicity in male and female rats

	Treatment		Organ weight		Serum	
	PB (mg/kg)	BHT (mg/kg)	Liver (g/100g BW)	Kidney (g/100g BW)	GPT (IU/l)	UN (mg/dl)
Males						
	−	0	4.32 ± 0.10	0.768 ± 0.030	29 ± 5	24.1 ± 1.0
	−	500	4.43 ± 0.16	0.765 ± 0.017	39 ± 10	22.5 ± 1.2
	−	1,000	4.21 ± 0.08	0.788 ± 0.022	220 ± 50*	26.3 ± 2.8
	+	0	4.68 ± 0.20	0.763 ± 0.022	29 ± 3	24.6 ± 1.1
	+	500	4.99 ± 0.06*	0.813 ± 0.026*	4,390 ± 1,900*	26.1 ± 2.3
	+	1,000	6.14 ± 0.42*	0.990 ± 0.084*	10,000 ± 3,500*	86.4 ± 12.3*
Females						
	−	0	3.75 ± 0.10	0.800 ± 0.022	27 ± 3	22.9 ± 0.2
	−	500	3.85 ± 0.14	0.808 ± 0.015	27 ± 2	28.2 ± 2.8
	−	1,000	3.97 ± 0.07*	0.803 ± 0.034	40 ± 17	21.7 ± 4.0
	+	0	4.61 ± 0.05	0.810 ± 0.022	28 ± 3	25.0 ± 1.8
	+	500	4.70 ± 0.14	0.818 ± 0.039	1,940 ± 940*	27.8 ± 2.8
	+	1,000	5.15 ± 0.40*	0.833 ± 0.022	5,710 ± 2,480*	29.9 ± 10.3

Animals pretreated with PB (80 mg/kg, i.p., 4 times) or saline were killed 24 hrs after oral administration of BHT. Each value represents the mean ± S.D. from five rats.

* Significant difference from the corresponding control values ($p < 0.05$)

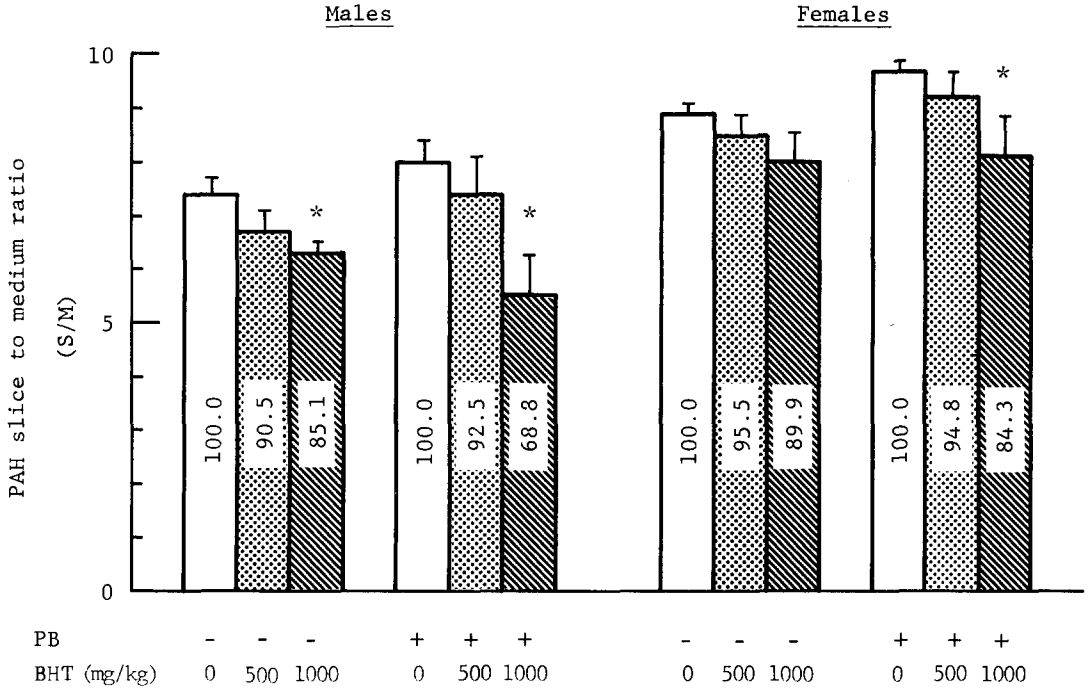

Fig. 1. Effect of BHT and/or phenobarbital (*PB*) treatment on PAH accumulation by renal slices in vitro. Female and male rats pretreated with PB (80 mg/kg, ip, daily for 4 days) or saline were killed 24 h after oral administration of BHT. Each value represents the mean ± SD from five rats. Value in each bar represents percentage of corresponding control. * $p < 0.05$

duced a decrease in the accumulation of PAH by kidney slices in vitro (Fig. 1). The decrease in PAH accumulation was intensified by PB pretreatment, as shown by the figures in each bar.

The results in Table 1, and Figs. 1 and 3A, B indicate that BHT-induced renal damage in male rats is greater than in female rats. Because of this observation, male rats were used in subsequent experiments.

Figure 2 shows serum GPT activity and serum UN level plotted against time after PB and/or BHT administration to male rats. The levels of serum UN were unaffected by the doses of BHT alone throughout the experimental

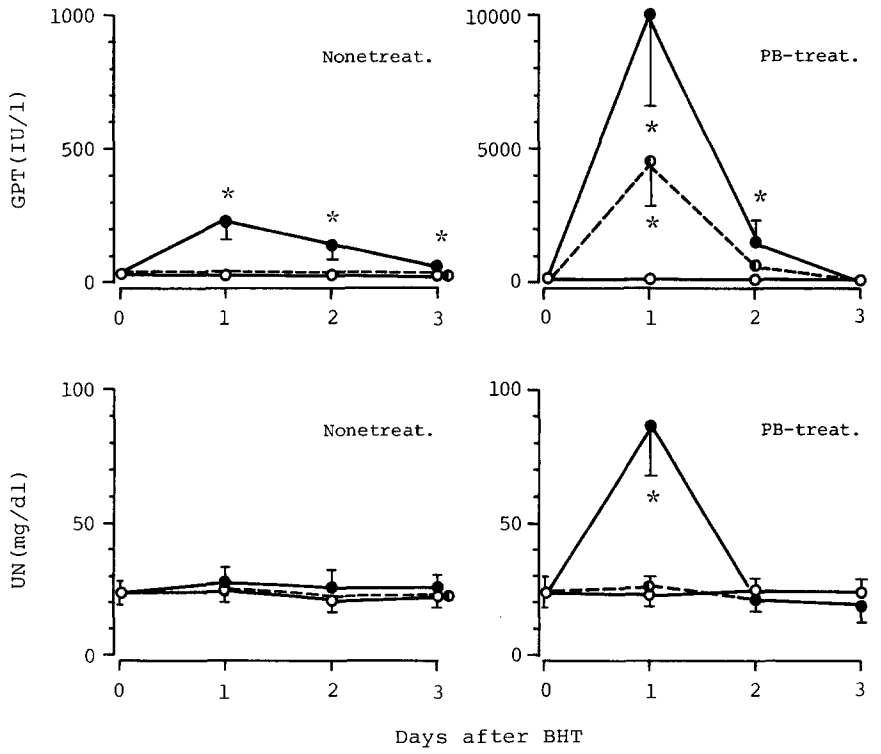

Fig. 2. Time-course of changes in the activity of serum GPT and in the level of serum UN after oral administration of BHT [dose: 0 (○), 500 (◐), 1,000 (●) mg/kg] to male rats pretreated with phenobarbital (*PB-treat*) or saline (*Nonetreat*). Each value represents the mean ± S.D. from five rats. * Significantly different from control, $p < 0.05$

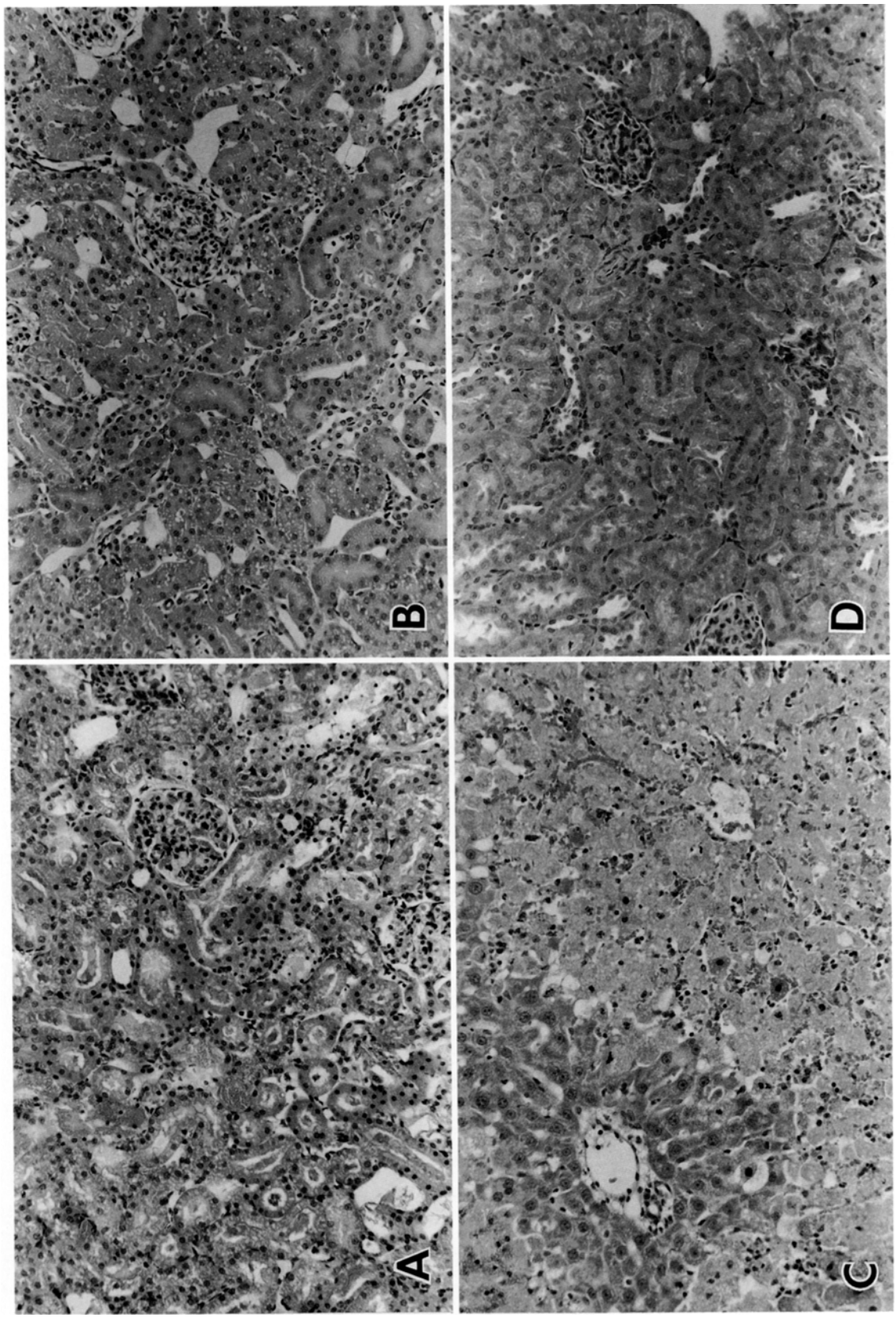

Fig. 3. Kidney and liver sections after treatment with BHT (1,000 mg/kg) from rats pretreated with phenobarbital. (**A**) kidney of male (24 h after BHT): renal tubular necrosis is seen, (**B**) kidney of female (24 h after BHT): no obvious alteration in renal tubules is seen, (**C**) liver of male (24 h after BHT): centrilobular necrosis is seen, (**D**) kidney of male (3 days after BHT): no alteration in renal tubules is seen Magnification: 200 x

period. The levels in PB plus high-dose BHT-treated male rats, however, reached a maximum at 24 h, the same time at which the kidney exhibited slight renal tubular necrosis (Fig. 3A). After that time, the levels of serum UN and the histological changes were rapidly reversed (Figs. 2 and 3D). Even 3 days after high-dose BHT administration to PB-pretreated rats, the reduced accumulation of PAH had not yet returned to control levels (85% of BP-positive control, data not shown). The elevation of serum GPT activity induced by BHT alone or PB plus BHT was rapidly reversible in this experimental period.

Figures 4 and 5 show the results of urinalyses. Polyuria, proteinuria and enzymuria (except for AP) with a concomitant decrease in urine specific gravity (data are not shown), were observed at 24 and 48 h after high-dose BHT treatment. Further, pretreatment with PB resulted in an increase in urine volume and excretion of urinary protein and enzymes.

The results in Figs. 4 and 5 lend biochemical support to BHT-induced renal failure. Since hematuria, assayed by Labstix-III (Miles-Sankyo Co. Ltd, Japan), was found in PB plus high-dose BHT-treated male rats, part of the renal damage may be due to glomerular abnormalities (Fig. 3).

Discussion

Although few studies have evaluated the effects of BHT on renal functioning, in the present study we have demonstrated that a single large dose of BHT causes some renal damage in male rats. The renal damage was demonstrable biochemically by reduced accumulation of PAH by renal slices, and by proteinuria and enzymuria. Pretreatment of rats with PB potentiated the nephrotoxicity of BHT. This conclusion was confirmed histologically by distal tubular necrosis. The kidney may be a target organ for the toxic action of a large dose of BHT.

In previous studies, sex differences were found in BHT-induced renal damage. Some kidney injury, especially in the distal tubule of the loop of Henle, was noted in female rats fed a semisynthetic diet supplemented with 1% BHT for 4 weeks (Meyer et al. 1978). In our present experiment with PB pretreatment, a sex difference in susceptibility to BHT-induced renal damage (assessed by serum UN) was observed. Male rats were more sensitive than the female rats (Table 1, Fig. 3). This sex difference was also evidence by the difference in the reduced degree of PAH accumulation by renal slices between males and females (Fig. 2). Further, this same predisposition was found in the hepatotoxicity induced by BHT alone or PB plus BHT (Table 1). Based on these results, it may be concluded that male rats are more susceptible to BHT-induced nephrotoxicity and hepatotoxicity than female rats.

The kidney is exposed not only to parent compound, but to a variety of hepatic metabolites as well (Ladomery et al. 1967). Previously, our studies have demonstrated that BHT is metabolized to BHT-quinone methide (putative toxic metabolite) by a cytochrome P-450-linked monooxygenase system, and that the 4-methyl group of the metabolite specifically binds to the sulfhydryl group of protein, glutathione and cysteine (Nakagawa et al. 1981, 1983). Further, we found that the acute hepatic damage, centrilobular necrosis, induced by BHT was associated with a remarkable depletion of glutathione and an elevation in the amount of activated intermediate bound to protein (Nakagawa et al. 1981, 1983). Following administration of ^{14}C-labeled BHT to rats, the covalently bound radioactive metabolite was concentrated in liver and kidney (Nakagawa et al. 1979a). For example, 24 h after oral administra-

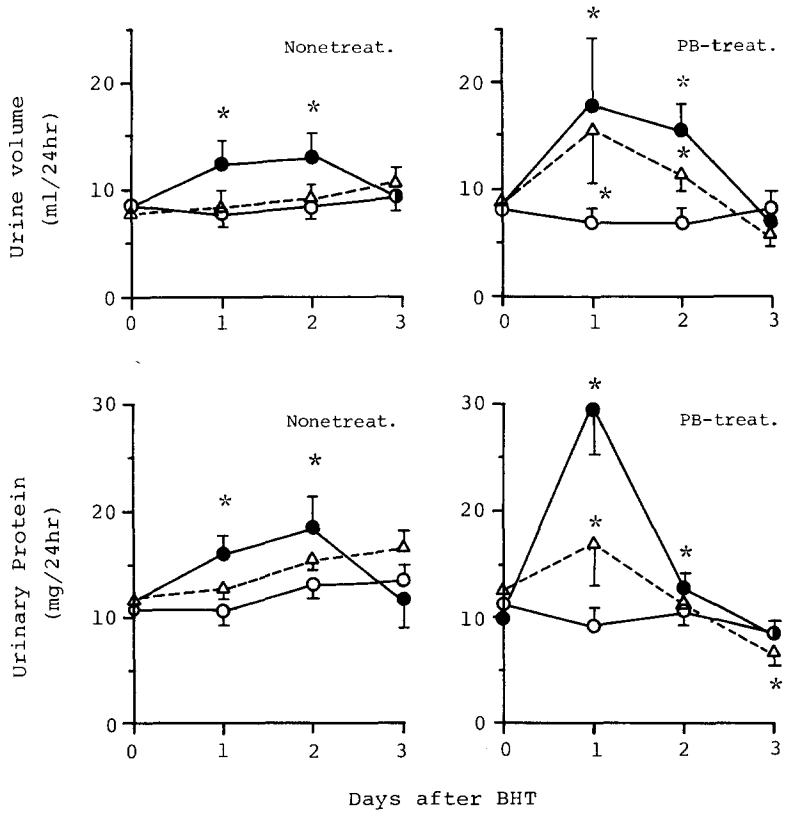

Fig. 4. Effect of BHT and/or phenobarbital (*PB*) treatment on urine volume and urinary protein. Male rats pretreated with PB (*PB-treat*) or saline (*None-treat*) were dosed with BHT [dose: 0 (○), 500 (△), 1,000 (●) mg/kg]. Each value represents the mean ±s.d. from five rats. * Significantly different from control, $p < 0.05$

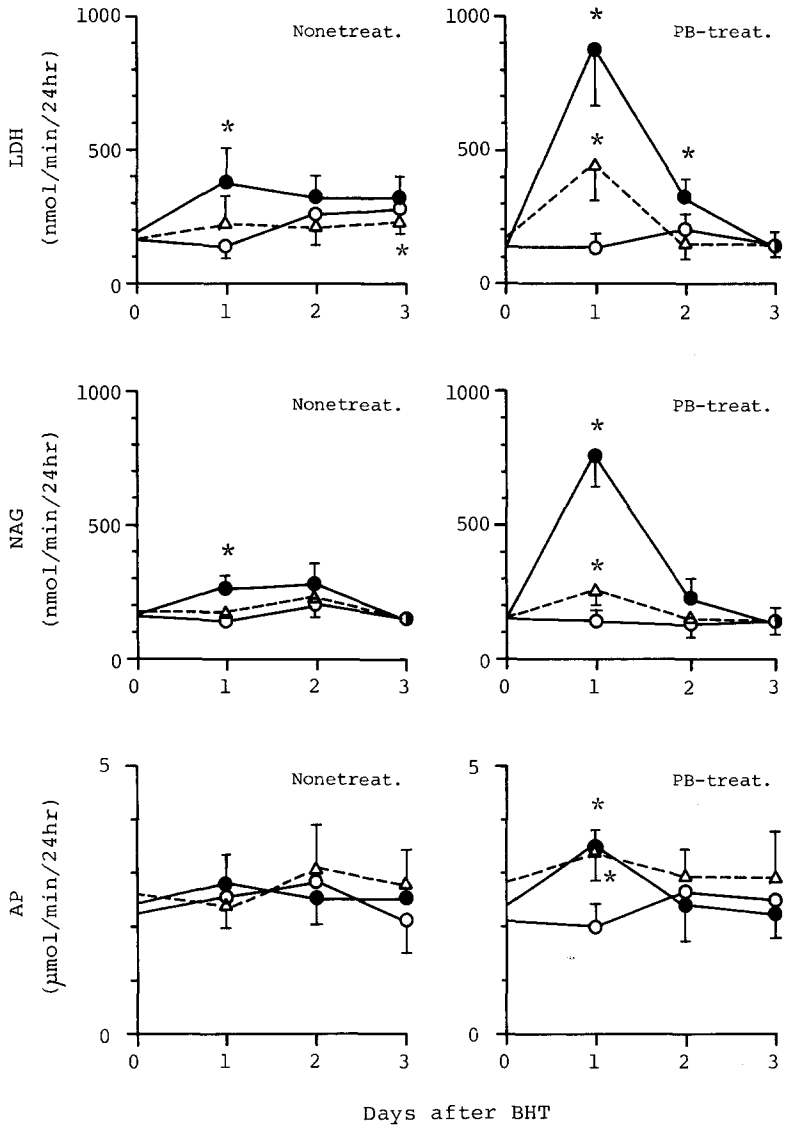

Fig. 5. Effect of BHT and/or phenobarbital treatment on the urinary excretion of lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (*NAG*) and alkaline phosphatase (*AP*). Male rats pretreated with PB or saline were dosed with BHT [dose: 0 (○), 500 (△), 1000 (●) mg/kg]. $p < 0.05$

tion of BHT to rats, the amount of metabolite bound to kidney protein was about 60% of that bound to liver protein.

However, the metabolizing and binding capacity of BHT in kidney microsomes was only about 6% of that in liver microsomes (Nakagawa et al. 1979b). Recent studies have made it clear that activated metabolites of bromobenzene, naphthalene, 1,1-dichloroethylene, and other compounds are sufficiently stable to diffuse out of cells and be carried to organs throughout the body where they can bind covalently and can exert toxic effects (Gram et al. 1986). On the other hand, Ford et al. (1978) reported that organic ion transport, which is an index of renal function, was reduced with the addition of BHT alone to the incubation mixture. It is well known that PB does not affect renal mixed-function oxygenase activity in the rat (Ohnhaus and Siegel 1974). Further, PB increases renal blood flow and urinary excretion of xenobiotics, in addition to the induction of hepatic monooxygenase and conjugation systems (Ohnhaus 1972). Therefore, the present results suggest that BHT is metabolically altered within the liver, and that the some activated metabolite (BHT-quinone methide) transported by blood induces cytotoxicity in the kidney. Of course, part of the depressed PAH accumulation may be attributed to the parent compound.

References

Bradford M (1976) A rapid and sensitive method form the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Cross RJ, Taggart VJ (1950) Renal tubular transport: Accumulation of *p*-aminohippurate by rabbit kidney slices. Am J Physiol 161: 181–190

Daniel JW, Gage JC (1965) The absorption and excretion of butylated hydroxytoluene (BHT) in the rat. Food Cosmet Toxicol 3: 405–415

Daniel JW, Gage JC, Jones DI (1968) The metabolism of 3,5-ditert-butyl-4-hydroxytoluene in the rat and in the man. Biochem J 106: 783–790

Fawcett JK, Scott JE (1960) A rapid and precise method for the determination of urea. J Clin Pathol 13: 156–159

Ford SM, Hook JB, Bond JT (1980) The effects of butylated hydroxyanisole and butylated hydroxytoluene on renal function in the rat. II Effects on organic acid and base transport. Food Cosmet Toxicol 18: 21–26

Gram TE, Okine LK, Gram RA (1986) The metabolism of xenobiotics by certain extrahepatic organs and its relation to toxicity. In: George R, Okun R, Cho AK (eds) Ann Rev Pharmacol Toxicol 26. Annual Reviews Inc, California, pp 259–291

Horak E, Hopfer SM, Sunderman FW (1981) Spectrophotometric assay for urinary N-acetyl-β-D-glucosaminidase activity. Clin Chem 27: 1180–1185

Karmen A (1955) A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. J Clin Invest 34: 131–133

Karplyuk IA (1960) Elementary raw fats containing phenolic antioxidants. An elevation from the point of view of hygiene. Vopr Pitaniya 19: 67

Kind PPN, King EJ (1954) Estimation plasma phosphatase by determination of hydrolyzed phenol with aminoantipyrine. J Clin Path 7: 322–326

Ladomery LG, Ryan AJ, Wright SE (1967) The excretion of (^{14}C) butylated hydroxytoluene in the rat. J Pharm Pharmacol 19: 383–387

Meyer O, Blom L, Olsen P (1978) Influence of diet and strain of rat on kidney damage observed in toxicity studies. Arch Toxicol suppl 1: 355–358

Mizutani T, Nomura H, Nakanishi K, Fujita S (1987) Hepatotoxicity of butylated hydroxytoluene and its analogs in mice depleted of hepatic glutathione. Toxicol Appl Pharmacol 87: 166–176

Nakagawa Y, Hiraga K, Suga T (1979a) Biological fate of butylated hydroxytoluene (BHT); binding in vivo of BHT to macromolecules of rat liver. Chem Pharm Bull 27: 442–446

Nakagawa Y, Hiraga K, Suga T (1979b) Biological fate of butylated hydroxytoluene (BHT); binding in vitro of BHT to liver microsomes. Chem Pharm Bull 27: 480–485

Nakagawa Y, Hiraga K, Suga T (1981) Biological fate of butylated hydroxytoluene (BHT); binding of BHT metabolites to cysteine in vitro. Biochem Pharmacol 30: 887–890

Nakagawa Y, Hiraga K, Suga T (1983) On the mechanism of covalent binding of butylated hydroxytoluene to microsomal protein. Biochem Pharmacol 32: 1417–1421

Nakagawa Y, Tayama K, Nakao T, Hiraga K (1984) On the mechanism of butylated hydroxytoluene-induced hepatic toxicity in rats. Biochem Pharmacol 33: 2669–2674

Nakagawa Y, Tayama K, Nakao T, Hiraga T (1985) Effect of cobaltous chloride on butylated hydroxytoluene-induced hepatic necrosis in rats. Toxicol Lett 24: 85–89

Newton JF, Kuo CH, Gembory NW, Mudge GH, Hook JB (1982) Nephrotoxicity of *p*-aminophenol, a metabolite of acetaminophen, in the Fischer 344 rat. Toxicol Appl Pharmacol 65: 336–344

Ohnhaus EE (1972) Urinary excretion of chlorothiazide in rats before and after phenobarbitone administration. Experientia 28: 821–822

Ohnhaus EE, Siegl H (1974) Changes in renal function following chronic phenobarbitone administration. Br J Pharmacol 52: 141

Powell CJ, Connelly JC, Jones SM, Grasso P, Bridges JW (1986) Hepatic responses to the administration of high doses of BHT to the rat: their relevance to hepatocarcinogenicity. Food Chem Toxicol 24: 1131–1143

Smith HW, Finkelstein N, Aliminosa L, Crawford B, Graber M (1945) The renal clearance of substituted hippuric acid derivatives and other aromatic acids in dog and man. J Clin Invest 25: 388–404

WHO Technical Report Series (1987) Evaluation of certain food additives and contaminants. Thirtieth Report of the Joint FAD/WHO Expert Committee on Food Additives, Geneva, pp 12–52

Wroblewski F, LaDue JS (1955) Lactic dehydrogenase activity in blood. Proc Soc Exp Biol Med 90: 210–213

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