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EFFECTS OF BHA AND ACETAMINOPHEN ON THE BILIARY EXCRETION OF PHENOLPHTHALEIN AND THE HEPATIC GLUCURONIDATION IN MALE RATS

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ABSTRACT: *The present study examined the effects of butylated hydroxyanisole (BHA) on acetaminophen (AA)-induced hepatotoxicity in male rats and also examined the effects of these compounds on the biliary excretion of phenolphthalein (PP) and the hepatic glucuronidation. Male Sprague-Dawley rats were pretreated with BHA (0.75% in diet for 10 days) were given single dose of AA (600 mg/kg, ip) and liver function was determined 24 hr later. Serum activity of alanine aminotransferase (ALT) and histopathology were used as indices of hepatotoxicity. BHA pretreatment remarkably decreased ALT activity relative to control values from rats treated with AA alone, and slightly decreased hepatic centrilobular necrosis produced by AA. BHA partially prevented AA-induced hepatotoxicity. Also, the plasma disappearance and biliary excretion of PP were increased in BHA-pretreated rats as well as rats treated with AA. To investigate this phenomenon, UDP-glucuronyltransferase (UDPGT) activity and hepatic UDP-glucuronic acid (UDPGA) concentration were determined. Hepatic UDPGA concentrations in BHA-pretreated and AA-treated rats were significantly higher than control rats. UDPGT activity in BHA pretreated rats was also significantly higher than control, whereas the activity in AA-treated rats was not shown significant changes from control. In conclusion, BHA pretreatment partially protected against AA-induced hepatotoxicity in male rats. The mechanism of this protection involves an increased detoxication by enhanced glucuronidation of AA. Also, the present study suggested that the increased plasma disappearance and biliary excretion of PP which is shown after BHA pretreatment and/or*

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AA injection appear to be directly related to hepatic UDPGA concentration rather than UDPGT activity.

Key Words: *BHA, Acetaminophen, Phenolphthalein, Glucuronidation, Rats.*

INTRODUCTION

Acetaminophen (AA=paracetamol) is widely used analgesic and antipyretic drug which produces hepatotoxicity in both humans and laboratory animals at excessive dosage (Boyd and Bereczky, 1966; Davison and Eastham, 1966; Hinson, 1982; Black, 1984). Although a single large dose of AA produces the same type of lesions in the liver of a variety of laboratory animals (Davies *et al.*, 1974) and in humans (Prescott *et al.*, 1971), there are considerable differences in the susceptibility of varies species to AA toxicity. Hamsters and mice are among the most sensitive species, whereas rats and guinea pigs are remarkably resistant (Davies et. al., 1974; Green *et al.*, 1984; Gregus *et al.*, 1988).

All species metabolize the majority of AA to nontoxic sulfate and glucuronide conjugates (Coldwell *et al.*, 1976) and oxidize a smaller fraction of the dose to an electrophilic metabolite, probably N-acetyl-p-benzoquinoneimine (Hinson, 19 82; Dahlin *et al.*, 1984; Vermeulen *et al.*, 1992). It is thought that species variation in the biotransformation of AA is a major determinant of the species differences in sensitivity toward AA-induced liver injury. So, susceptibility to AA-induced hepatotoxicity depends not only on the rate of formation of the toxic metabolite, but also on the rate of the detoxication pathway reaction, glucuronidation and sulfation. Recently, Gregus *et al.* (1988) found that rats excreted AA-glucuronide and AA-sulfate into bile at a higher rate than the other susceptible species.

Butylated hydroxyanisole (BHA) is a food antioxidant that possesses a variety of properties of toxicologic interest. This compound has been shown to reduce the carcinogenic and hepatotoxic effects of several structurally diverse compounds such as polycyclic hydrocarbons, halogenated solvents, drugs, and plant toxins (Wattenberg, 1978; Miranda *et al.*, 1981; Kim and Jones, 1982; Ansher *et al.*, 1983). The protective effects of BHA are thought to be largely due to its ability to increase the activities of several enzymes involved in the detoxification of various toxic or carcinogenic metabolites. BHA increases hepatic and extrahepatic activities of glutathione S-transferase (s) and epoxide hydrolase, and elevates nonprotein sulfhydryl content (Benson *et al.*, 1978, 1979; Cha *et al.*, 1978).

Also, an increase in an additional phase II biotransformation pathways has been reported to occur in mice following BHA feeding. The activity of mouse hepatic UDP-glucuronyltransferase (UDPGT) was significantly increased following BHA treatment (Cha and Bueding, 1979; Cha and Heine, 1982). Hazelton *et al.* (1985) have demonstrated that BHA increased the *in vitro* capacity for glucuronidation in mice by elevating both UDPGT activities and UDPGA concentration in liver. They have also demonstrated that BHA prevented AA-induced hepatotoxicity in mice, and enhanced the rate of urinary elimination of AA, and they also concluded that the increase in AA glucuronidation during BHA feeding to mice play a role

in the enhanced excretion of AA as well as protection against AA-induced hepatotoxicity (Hazelton *et al.*, 1986).

Several aspects pertaining to the effects of BHA ingestion on AA-induced hepatotoxicity and glucuronidation capacity were examined in the present study. First, because previous investigations have examined the effects of BHA treatment on AA-induced hepatotoxicity in mouse only, possible species-dependent differences in response to BHA and AA were examined. Second, because it is known that AA injection decreases the activity of mouse hepatic UDPGT (Huh *et al.*, 1987) and UDPGA concentration (Hazelton *et al.*, 1986), and that BHA increases the *in vitro* capacity for glucuronidation in mice by elevating both UDPGT activities and UDPGA concentration (Hazelton, *et al.*, 1985), next aim was to study the effects of BHA and AA on the biliary excretion of phenolphthalein (PP), which is commonly used to test liver function and is conjugated with glucuronic acid before its biliary excretion (Clark and Cooke, 1978; Gregus, *et al.*, 1983; Hickey *et al.*, 1991). Finally, the effects of BHA and AA on glucuronidation capacity reflected by UDPGT activities, UDPGA concentration in liver were examined.

MATERIALS AND METHODS

Materials

Butylated hydroxyanisole (BHA), acetaminophen (AA), phenolphthalein (PP), Trizma base, diethylstilbestrol (DES), bovine liver β-glucuronidase (Type B1), and Brig 58 (polyethylene 20-cetyl ether), UDP-glucuronic acid (UDPGA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma Chemical Company, St. Louis, MO; [monoethyl-^{3}H]-DES (79 Ci/mmol) was purchased from Amersham Life Science, Amersham, U.K.; [G-^{3}H]Acetaminophen (1.26 Ci/mmol) was obtained from New England Nuclear Products (Boston, Mass). All other chemicals were the highest quality available.

Pretreatment of Rats

Male Sprague-Dawley rats, 250~300 g, were pretreated as follows: Rats were fed a diet containing BHA (0.75%) for 10 days. AA (600 mg/kg) was injected intraperitoneally (ip) 24 hr before the experiment. 0.1 N NaOH/Saline (final pH 10.8) as controls was administered ip.

Hepatotoxicity Studies

The toxicity produced by AA was assessed 24 hr after injection (ip). Elevated plasma alanine aminotransferase (ALT) activity and liver histopathology were used as indices of liver damage. Blood was collected by decapitation and serum was prepared for measurement of ALT (Sigma Kit No. 59-UV). For histopathological studies, tissue from the median lobe of the liver (approximate 3 mm thick) was removed from all rats and was fixed in 10% buffered formalin. Sections (6 μm thick) were mounted on slides and stained with hematoxylin and eosin, and examined by two observers by light microscopy by the method of Chalkley (1943) as described by Madhu and Klaassen (1991).

Biliary Excretion Studies

For the biliary excretion studies, rats were anesthetized with urethane (1.0 g/kg, 4 ml/kg, ip). The common bile duct, femoral vein and femoral artery of the rats were cannulated using PE tubing of the appropriate size. The venous cannula was used for injections while the arterial cannula served for the collection of blood samples. PP (50 mg/kg, 2 ml/kg) was injected into the femoral vein. PP was dissolved in 20% ethanol-80% propylene glycol. Bile was collected for six consecutive 10-min periods after injection of PP. The volume of bile collected was measured gravimetrically assuming a specific gravity of 1.0 g/ml. Bile flow was calculated as microliters per minute per gram of wet liver weight. Biliary excretion rates were calculated as the product of bile flow and bile concentration. To estimate the plasma disappearance of PP, 250 μl blood samples were taken into heparinized tubes from the femoral artery at 1, 5, 10, 20, 40 and 60 min after its administration. Body temperature of rats was maintained at 37℃ during anesthesia.

Total PP (unconjugated plus conjugated) in plasma and bile was measured spectrophotometrically at 550 nm on a Shimazu Model UV-120-2 spectrophotometer (Kyoto, Japan) after incubation with β-glucuronidase and subsequent dilution with 0.3 M glycine buffer, pH 10.4 (Clark and Cooke, 1978), whereas unconjugated PP was measured directly without hydrolysis. Concentration of conjugated PP was obtained as the difference between total and unconjugated.

Tissue Preparation

After 10 days of BHA treatment the rats were decapitated. For analysis of UDPGA concentration, 0.5 g portion of liver was placed in a test tube with 2.0 ml of distilled water. The sample was placed in a boiling water bath for 3 min, homogenized, and centrifuged at 20,000×g for 20 min at 4℃. Liver samples for enzymatic analysis were homogenized (25% w/v) in ice-cold 0.25 M sucrose with a glass Potter-Elvehjem mortar and a Teflon pestle. The homogenate was centrifuged at 10,000×g for 10 min at 4℃. The resultant supernatant fraction was centrifuged at 105,000×g for 65 min for microsomal fraction. The microsomal pellet was resuspended in 0.25 M sucrose (0.33 g equivalent wet weight of liver/ml 0.25 M sucrose). A portion of this suspension was diluted with an equal volume of 0.25 M sucrose and resultant preparation was termed "native" microsomes. A second portion of the 33% suspension was diluted with an equal volume of 0.25 M sucrose containing 16 mM CHAPS and agitated for 20 min at 4℃. This preparation was termed "activated" microsomes. Protein concentrations were determined by the method of Lowry *et al.* (1951).

UDP-glucuronyltransferase (UDPGT) Activities

UDPGT activities were assayed toward the following acceptors in both native and activated microsomal preparations. Enzyme activity was determined by spectrophotometric and radiometric methods with the following aglycone concentration: 0.5 mM 4-nitrophenol (4-NP; Moldeus, *et al.*, 1976), 0.15 mM PP (Winsnes, 1969), and 5 mM AA (Hazelton, *et al.*, 1985). All assays (0.5 ml) were performed at 37℃ and the 0.5 ml incubation mixture contained 750 μg of protein (300 μl) of hepatic microsomes; 0.2 M Tris-HCl (pH 7.5), 2.2 mM saccharic acid-1,4-lactone, and 10

mM $MgCl_2$. Reactions were started by the addition of UDPGA (final concentration 4 mM) and terminated after 20 min incubation period except for 4-NP incubation with activated microsome which was incubated for 3 min. Reagent blanks contained no UDPGA. Glucuronidations of 4-NP and PP were determined spectrophotometrically at 400 nm and 550 nm, respectively. Radioactivity was determined radiometrically by liquid scintillation spectroscopy using complete cocktail OptiPhase 'Hisafe' 3 (LKB Scintillation Products) and Wallac 1410 Liquid Scintillation Counter (Wallac Oy, Finland).

Enzymatic Determination of UDPGA

Hepatic UDPGA concentration was determined by the method of Watkins and Klaassen (1982) in which UDPGA is quantitated via a UDPGT-catalyzed reaction with [^{3}H]diethylstilbestrol as the acceptor substrate.

Statistical analysis

Results were expressed as the mean± S.E. The significance of the difference between mean values was assessed by the Student's t test ($p<0.05$).

RESULTS

The initial approach of this investigation was to establish that BHA protected against AA-induced hepatotoxicity in male rat. AA was administered at a dosage of 600 mg/kg, ip, and the indices of liver damage (i.e., elevated plasma ALT activity, and liver histopathology) were assessed 24 hr after AA injection. Throughout this investigation no lethality was found in all rats. However, administration of AA produced severe internal hemolysis and BHA-pretreatment hardly exerted the protective effects on the hemolysis (data not shown).

The effect of BHA on plasma enzyme activity is shown in Table 1. Injection of AA increased ALT activity 3-fold when compared to control rats. However, when rats fed BHA were administered AA, slight increase in plasma enzyme activity was observed.

The quantitative effect of BHA on AA-induced hepatotoxicity was also studied by histopathological examination (Table 2). The criteria for quantitation of the histopathology is described under MATERIALS AND METHODS. BHA pretreatment decreased the histopathological changes produced by AA, which confirms the protection noted by the biochemical measurement (Table 1). Therefore, based on above results, BHA pretreatment partially protected against AA-induced hepatotoxicity.

The next experiments were to study the effects of BHA and AA on the plasma disappearance and biliary excretion of PP, which are conjugated with glucuronic acid before its biliary excretion. Figure 1 shows the effect of BHA and AA on plasma disappearance of unconjugated PP. In general, there was a tendency for plasma unconjugated PP concentration to be lower in BHA-pretreated rats. Also AA injection further increased the plasma disappearance of PP. Figure 2 shows the effect of BHA and AA on plasma disappearance of conjugated PP. Concentration of conjugated PP was higher than that of control in 1, 5, 10 min but lower

Table 1. Effect of BHA on Acetaminophen-induced ALT activity.[a]

Treatment	ALT Activity (units/ml)
Control	36.8± 2.5
Acetaminophen[b]	150.1± 12.8[d]
BHA(0.75%)	42.9± 2.3
BHA(0.75%)+Acetaminophen[c]	83.6± 6.5[d]

[a]. Values are expressed as the mean ± SE of 5 rats. [b]. AA (600 mg/kg) was injected ip 24 hr before the experiment. [c]. Fed a diet containing 0.75% BHA for 10 days before the experiment. [d]. Values significantly different from control ($p<0.05$).

Table 2. Effect of BHA on Acetaminophen-Induced Hepatic Necrosis.[a]

			Extent of Necrosis[a]				
Pretreatment	AA(mg/kg)	N	0	1+	2+	3+	4+
Control	0	12	100	0	0	0	0
	600	12	0	0	12	64	24
BHA[b]	0	9	100	0	0	0	0
	600	9	0	15	50	30	5

[a]. Extent of hepatic necrosis was scored by the criteria given under MATERIALS AND METHODS.
[b]. Fed a diet containing 0.75% BHA for 10 days.

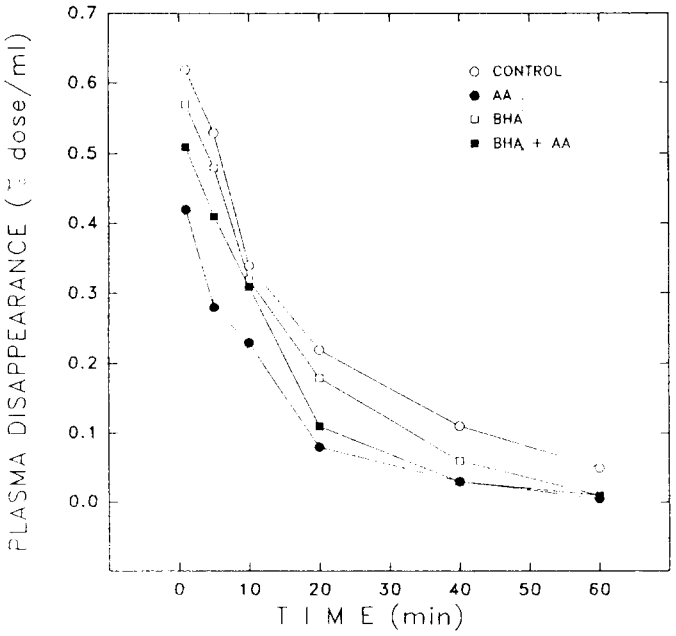

Figure 1. Effect of BHA and AA on the Plasma Disappearance of PP.

in 20, 40, and 60 min sample.

Figure 3 shows the effect of BHA on the biliary excretion of PP. Biliary excretion of PP was markedly increased in rats pretreated with BHA. Also AA injection fur-

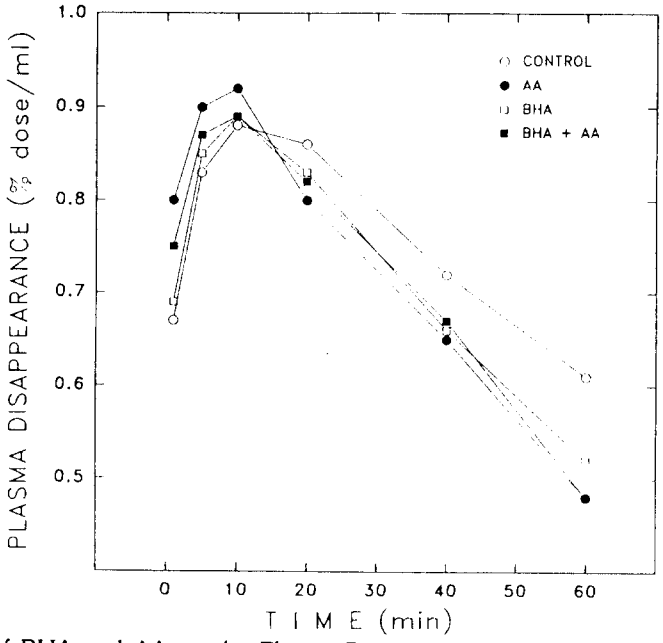

Figure 2. Effect of BHA and AA on the Plasma Disappearance of PPG.

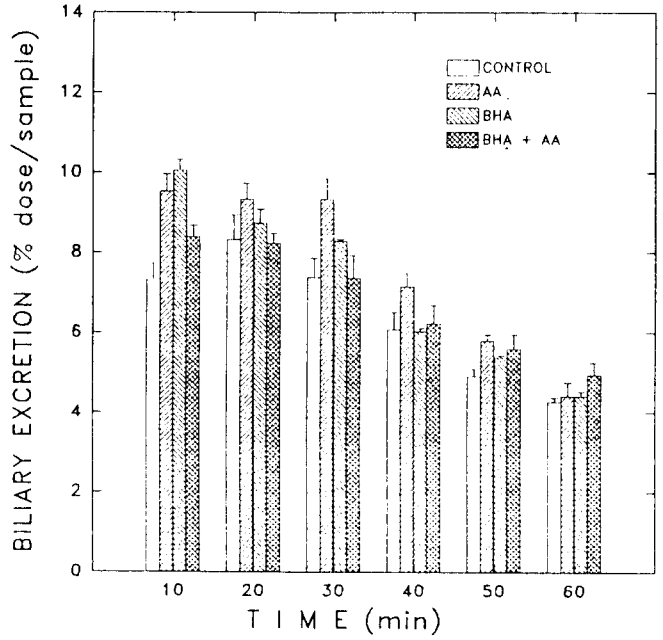

Figure 3. Effect of BHA and AA on the Biliary Excretion of PP.

ther incresed the biliary excretion of PP. More enhancement of plasma disappearance and biliary excretion of PP was observed in AA-injected rats than BHA treated rats.

Table 3 shows the effect of BHA and AA on the liver weight, bile flow and

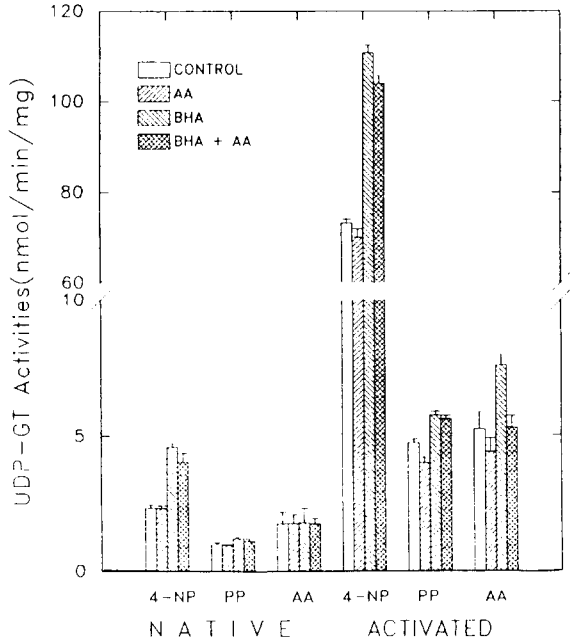

Figure 4. Effects of BHA and Acetaminophen (AA) on Hepatic UDP-glucuronyltransferase (UDPGT) activity.

Table 3. Effect of BHA and Acetaminophen on Hepatic Functions.[a]

Treatment	Liver weight (% body wt.)	Bile flow (mg/min/g liver)	Hepatic Microsomal Protein (mg/g liver)
Control	3.27± 0.08	2.41± 0.06	22.1± 0.95
Acetaminophen (AA)[b]	3.26± 0.06	3.10± 0.26*	26.9± 1.19*
BHA (0.75%)	4.11± 0.14*	2.52± 0.05	23.9± 0.72
BHA (0.75%)+AA[c]	3.87± 0.03*	2.79± 0.06*	23.2± 0.85

[a]. Values are expressed as the mean ± SE of 9 rats. [b]. AA (600 mg/kg) was injected ip 24 hr before the experiment. [c]. Fed a diet containing 0.75% BHA for 10 days before the experiment. * Values significantly different from control ($p<0.05$).

Table 4. Effects of BHA and Acetaminophen (AA) on Hepatic UDP-glucuronic acid (UDPGA) cencentration[a].

	UDP-glucuronic acid concentration (nmol/g of liver)	
	Native microsome	Activated microsome
Control	230± 11.6	510± 30.0*
AA	343± 16.9*	1626+70.3*
BHA	275+18.0*	1013+41.5*
BHA+AA	323+20.0*	1378+60.3*

[a]. Values are expressed as the mean± SE of 6 rats. [b]. AA (600 mg/kg) was injected ip 24 hr before the experiment. [c]. Fed a diet containing 0.75% BHA for 10 days before the experiment. *. Values significantly different from control ($p<0.05$).

the content of hepatic microsomal protein. BHA hardly affected on the content of hepatic microsomal protein. BHA pretreatment increased the liver weight, however AA injection increased the bile flow and the content of hepatic microsomal protein.

To study the increased biliary excretion of PP after AA injection, the effects of BHA and AA on glucuronidation capacity reflected by UDPGT activities and UDPGA concentration in liver were examined. UDPGT activities toward 4-NP, PP and AA in both native and activated microsomal preparations are shown in Figure 4. In control rats, the activities of UDPGT toward 4-NP, PP and AA in native microsomes was 2.35 ± 0.12, 1.01 ± 0.06, and 1.76 ± 0.43 nmol/min/mg, respectively. These activities could be activated 30-, 4-fold, and 3-fold with the detergent CHAPS. Activities in native and detergent-treated microsomal preparations from BHA-fed rats were significantly greater than the respective control values except toward AA in native microsome. Activities in native and detergent-treated microsomal preparations from AA-injected rats were similar or lower than the respective control values. Pretreatment with BHA significantly increased the activities toward 4-NP in AA-injected rats. However, pretreatment with BHA slightly increased the activities toward PP and AA in AA-injected rats.

Hepatic UDPGA concentrations in control and BHA-fed rats after AA injection are shown in Table 4. Although UDPGA concentration was significantly increased in BHA-pretreated rats, it was further increased in AA-injected rats.

DISCUSSION

Our initial evidence indicates that male rats fed BHA are partially protected against AA-induced hepatotoxicity. This evidence, based on plasma ALT activity and liver histopathology (Table 1 and 2), is in agreement with findings of other who also concluded that BHA pretreatment attenuates AA hepatotoxicity (Miranda *et al.*, 1983; Ansher *et al.*, 1983; Rosenbaum *et al.*, 1984; Hazelton *et al.*, 1986). However, in comparison with studies with other laboratory animals, the magnitude of hepatotoxicity in rats is appeared to be more or less lower than that of other animal studies. It is well known that rats are more resistant to AA-induced hepatotoxicity than other laboratory animals (i.e., mice, hamsters).

Susceptibility to AA-induced hepatotoxicity depends not only on the rate of formation of the toxic metabolite, but also on the rate of detoxication pathway reactions, glucuronidation and sulfation (Gregus *et al.*, 1988). Marked species variation in the AA-induced hepatotoxicity was documented. Rats, rabbits and guinea pigs are relatively resistant to AA-induced liver injury, and hamsters and mice are susceptible to AA-induced liver injury. The highest excretion of AA-glucuronide and detoxication pathway metabolites was found in the resistant species. Relatively small amounts of toxication pathway metabolites were excreted by species resistant to AA-induced liver injury (i.e., rats, rabbits, guinea pigs).

Miranda *et al.* (1983) reported that a sex difference was observed in the protective action of BHA against AA-induced hepatotoxicity in male and female mice. They reported that the protective effect of BHA against AA-induced hepatotoxicity was not demonstrated in male mice. In male, BHA pretreatment failed to block

the AA-induced increase in plasma ALT and the decrease in hepatic cytochrome P-450 content.

In general, glucuronidation plays a key role in the detoxification and excretion of xenobiotics and in the protection against AA-induced hepatotoxicity (Hazelton *et al.*, 1985, 1986). AA glucuronide and BHA-glucuronide is the predominant metabolite formed after high doses of AA and BHA, respectively (Hazelton *et al.* 1986; Astill *et 1al.*, 1962). Also PP is commonly used to test liver function, which is conjugated with glucuronic acid before its biliary excretion. Therefore, the effects of BHA and AA on the plasma disappearance and biliary excretion of PP were determined. Interestingly the plasma disappearance and biliary excretion of PP were increased in all rats treated with BHA or AA. However, more enhancement of plasma disappearance and biliary excretion of PP was observed in AA-injected rats than BHA treated rats. Gregus *et al.* (1983) suggested that PP was rapidly glucuronidated in the liver and probably in extrahepatic tissues as well. Also they insisted that conjugation with glucuronic acid was important for the transport of cholephils from blood to bile.

Because glucuronidation is dependent on UDPGT activity as well as intracellular UDPGA concentration, next experiment was undertaken to study the effects of BHA and AA on glucuronidation capacity reflected by UDPGT activities and UDPGA concentration in liver. As shown in Table 4 and 5, UDPGT activities and UDPGA concentration are elevated in BHA-treatment. The results of the present study indicate that the capacity of hepatic glucuronidation is elevated in rats on a diet supplemented with BHA. This conclusion is based on findings that both the activity of UDPGT toward specific substrates and the concentration of UDPGA are increased in liver of BHA-treated rats. In the present study, BHA treatment markedly increases UDPGT activities measured with 4-NP and PP. Also activities determined with AA are moderately enhanced. In addition to our findings, several other investigators have observed increases in the activities of UDPGT with other substrates in BHA-treated mice (Cha and Bueding, 1979; Cha and Heine, 1982; Rahimtula *et al.*, 1982; Hazelton *et al.*, 1985).

The present study demonstrates that AA injection markedly increases the UDPGA concentration in liver. The underlying biochemical mechanisms responsible for the enhancement of hepatic UDPGA concentration are unknown. It seems reasonable to assume that this high concentration of UDPGA is responsible for the relative resistance of rats to AA-induced hepatotoxicity. It would be more meaningful if the relationship between the UDPGA concentration (or formation) and resistance of other animals is determined. Also it is necessary to study the detailed time course of UDPGA concentrations after AA injection.

To our knowledge, this is the first experimental evidence indicating that AA injection markedly increases the plasma disappearance and biliary excretion of PP and hepatic UDPGA concentration. The findings of decreased UDPGT activity after AA dosing implies that the UDPGA concentration rather than UDPGT activity plays a key role in the glucuronidation and subsequent biliary excretion of PP.

In summary, the present findings indicate that male rats fed BHA are partially protected against AA-induced hepatotoxicity. Although BHA can elicit effects on several biochemical pathways such as glutathione conjugation and sulfation, its

ability to stimulate AA glucuronidation undoubtedly plays a key role in the protection against AA-induced hepatotoxicity. Also AA injection markedly increases the plasma disappearance and biliary excretion of PP and hepatic UDPGA concentration. The findings of decreased UDPGT activity after AA injection implies that the UDPGA concentration rather than UDPGT activity plays a key role in the glucuronidation and subsequent biliary excretion of PP.

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