Differences between the Antioxidation Effects of Butylated Hydroxyanisole and Butylated Hydroxytoluene on Acetaminophen - Induced Hepatic Metabolism in Rats

Jinhua Boindoigurong1*, Nasunsang2, Yukari Egashira1 and Hiroo Sanada1

1 Laboratory of Food and Nutrition, Graduate School of Science and Technology Chiba University (648 Matsudo, Matsudo-shi, Chiba 271-8510, JAPAN)
2 Mongolia Drugs Laboratory, Inner Mongolia Medical College (Hohhot-shi, Inner Mongolia, 010059, CHINA)

Abstract: The effects of dietary administration of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on acetaminophen (APAP)-induced hepatic metabolism in rats were examined. The administration method involves providing the rats with 0.5% each of BHA and BHT separately added to their diets for 7 days. Based on the results of the plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities for dietary administration, BHA and BHT in the diets fully prevented APAP-induced hepatotoxicity (500 mg / kg IP). By adding BHA and BHT to feedstuffs, hepatic UDP-glucuronosyltransferase (UDP-GTase) remained activated in the rats. The excreted amount of APAP-glucuronide increased and the residual APAP declined in the urine of the rats. After APAP administration in BHA or BHT pretreated rats, the excretion in plasma reached the largest amount for APAP-glucuronide at 2-4 h and APAP-sulfate at 6 h. It was clear that BHT excelled over BHA in playing the role of promoting hepatic metabolism. Data thus obtained showed the proposed different metabolisms in APAP-induced hepatotoxicity between BHA and BHT.

Key words: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), acetaminophen (APAP), hepatotoxicity, APAP-glucuronide, APAP-sulfate, rats

Introduction

Acetaminophen (APAP) is a drug widely used for analgesic and antipyretic purposes. It is generally considered as a safe drug; however, it may cause liver and kidney damage, even death after overdose. The metabolism of APAP involves conjugation in phase I and oxidation in phase II. The major excretion pathway for APAP is the formation of glucuronide and sulfate conjugated with the parent compound in phase I (1, 2). The conjugated compounds are formed by the cytochrome P-450 mediated oxidation of acetaminophen (3). Jollow et al. suggested that the toxicity of APAP was derived from a minor, yet highly reactive, N-acetyl-benzoquinoneimine (NAPQI) metabolite of APAP in phase II (4). The evidence that the NAPQI is reduced and detoxified by conjugation with glutathione (GSH) supports this proposition (5, 6).

Food antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), possess a
variety of properties of toxicologic interest. Many compounds have been shown to inhibit the carcinogenicity of a number of chemicals in laboratory animals (7-14). It has been reported in our foregoing work that both BHA and BHT have the function of protecting against the APAP-induced hepatotoxicity in the rats (15). The protective effects of BHA and BHT on the animals were well studied, however, the precise differences between BHA and BHT in the biochemical mechanisms underlying these effects are still unclear.

Hazelton et al. reported that BHA demonstrated its protective function against APAP-induced liver injury of mice through activation of UDP-glucuronosyltransferase (GTase) activity and increasing the APAP-glucuronide excretion (16). We have also proposed that BHA increased glutathione accumulation in the liver. On the other hand, BHT might induce an unknown compound X (such as certain kinds of proteins) that would conjugate with NAPQI, and the conjugates could protect rats from APAP-induced liver injury (17). The current work is focused on the protective functions and xenobiotic biotransformation pathways of BHA and BHT in APAP-induced hepatotoxicity.

2 Materials and Methods

2.1 Chemicals

APAP and APAP glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and an IATROZYME TA-LQ diagnostic kit was obtained from Iatron Co. (IATRON Laboratories, Inc., Tokyo, Japan) for plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement. BHA and BHT, phenyl methyl sulfonyl fluoride (PMSF), dithiothreitol (DTT), Brij 58, MgCl2, p-nitrophenol, UDP-glucuronic acid and other chemicals were supplied by Wako Pure Chemical Industries, Ltd., (Tokyo, Japan). APAP-sulfate with a potassium salt was synthesized according to the method of QI (18).

2.2 Animals and Experimental Design

Five-week-old male Wistar-SPF rats (SLC Co., Japan) were kept individually under 12 h light / dark cycles. The rats were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. They were given free access to water and commercial powdered food (CLEA CE − 2, Nippon CLEA, Tokyo, Japan) for 3 days and then to a standard diet (AIN-76 rodent diet) for 4 days. Composition of the standard diet is as follows (g / 100 g): Casein 20, Cornstarch 40, Sucrose 25, Corn oil 5, AIN76™ Vitamin (Nihon Nosan Kogyo KK) 1, AIN76™ Mineral (Nihon Nosan Kogyo KK) 3.5, Cellulose 5, Choline 0.2, and Methionine 0.3.

Experiment 1: The rats were divided into 3 groups of 15 each and placed in metabolic cages. For 7 days, each group was given an experimental diet containing BHA (0.5%) or BHT (0.5%) and the control group received the standard diet. In the case of the experimental diet, the total proportion of cornstarch and antioxidants were fixed at 40g / 100g of diet. These 3 groups were then treated with APAP.

The rats were fasted for 16 h before administering APAP at 0.5g / kg body weight intraperitoneally, and urine samples were then collected up to 24 h following the APAP treatment. At 6 h after APAP dosing, the rats were refed with their respective diets they had received previously, and at 24 h they were killed by decapitation. Blood and liver samples were collected. Aliquots of urine were stored at −20°C until analysis.

Experiment 2: The pretreatment is the same as in Experiment 1; rats were killed at 0, 2, 4, 6, 8, 24 h by decapitation. Blood samples were collected.

2.3 Analyses of Hepatic UDP-GTase Activity

Hepatic UDP-GTase activity in the microsomes was assayed according to the method of Bock et al. (19). The incubation mixture contains (mL / 0.50 mL): liver microsomes (1 mg protein / ml, 0.10), Brij 58 (0.25% w / v, 0.02), 1M Tris-HCl (pH 7.4, 0.05), 50 mM MgCl2 (0.05), 5 mM p-nitrophenol (0.05), H2O (0.18) and 30 mM UDP-glucuronic acid (0.05). After 2- min preincubation without UDP-glucuronic acid at 37°C, the reaction was started by adding UDP-glucuronic acid. At 0, 5, 10, 15, 20, 25, 30 min, 0.10 ml each of the incubation mixture was separated, and the protein was precipitated with 1 ml of 5% trichloroacetic acid. After centrifugation, 0.25 ml of 2 M NaOH was added to 1 ml of the supernatant, and the Glucuronidation of p-nitrophenol was determined spectrophotometrically at 405 nm.

2.4 Measurement of APAP and Its Metabolites

The supernatants of plasma or urine samples were prepared by adding perchloric acid according to the method of Wang (20) and centrifuged at 16,000 × g for
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10 min. These supernatants were analyzed for APAP and its metabolites by HPLC (Shimadzu LCMS, Shimadzu Seisakusho, Kyoto, Japan), with a variable wavelength visual / ultraviolet detector (model SPD-M10A). Separations were performed on a 25 cm × 4.6 mm ID (5-μm) Supelco, Discovery® RP Amide C18 column (Supelco, Inc., Bellefonte, PA). APAP and its metabolites were eluted with 7% acetonitrile (v/v in water) plus 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min and detected at 254 nm.

2.5 Statistical Analyses

Data are shown as means ± SE (standard deviation, N = 5 or 6). Data were analyzed by one-way ANOVA followed by Scheffe’s multiple comparison tests to determine significant differences between group means. The level of significance was taken as ** P < 0.01 or * P < 0.05.

3 Results

3.2 Effects of Dietary Pretreatment of BHA and BHT on Hepatic UDP-Glucuronosyltransferase Activity

Hepatic UDP-GTase catalyzes the conjugation of APAP with UDP-glucuronic acid. APAP-glucuronide is the predominant metabolite formed after APAP treatment. Hepatic UDP-GTase activities in the rats who ingested BHA or BHT for 7 days were 1.44-fold and 1.94-fold those of the APAP control group without the antioxidant, respectively (Table 1). In this study, activation of liver UDP-GTase was activated by continuous administration of BHA or BHT.

3.3 Effects of Dietary Pretreatment with BHA and BHT on the Decomposition of APAP and the Production of Its Major Metabolites in the Urine and Plasma of the Rats

Urine: urine was collected during 0-24 h after APAP administration APAP and its main metabolites, APAP-glucuronide and APAP-sulfate were then quantified by HPLC (Fig. 1). The excreted amounts of APAP-glucuronide in the rats fed with BHA or BHT were 2.5 and 3.7 times those of the APAP control group, respectively. However, the residual APAP amounts in the urine of the rats fed with BHA or BHT were 40% and 70% lower than that of the control group, respectively. No change in APAP-sulfate was observed among the three groups. We observed that the residual APAP amounts decreased accompanied by activation of the glucuronide pathway. BHA and BHT showed a considerable effect on the urinary excretion of APAP and its main metabolite, APAP-glucuronide.

Plasma: The livers of the rats which were fed with BHA or BHT for 7 days were examined at the certain times (0, 2, 4, 6, 8 and 24 h) after APAP treatment. The concentrations of APAP and its major metabolites in the

<table>
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<tr>
<th>Table 1</th>
<th>Effects of BHA and BHT on Hepatic UDP-GTase Activity of APAP Administered Rats.</th>
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<tbody>
<tr>
<td></td>
<td>UDP-GTase activity (mM / mg protein / min ± SE )</td>
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<tr>
<td>Control</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>BHA</td>
<td>0.52 ± 0.20*</td>
</tr>
<tr>
<td>BHT</td>
<td>0.70 ± 0.25**</td>
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Enzyme activities were determined with five rats in each group were given experimental diet as shown in 2.2 Experiment 1.
plasma were quantified by HPLC (Fig. 2). The maximum concentrations of residual APAP and APAP-glucuronide in the plasma were observed at 2 h after APAP treatment for all groups. The concentrations of APAP-glucuronide in the BHT- and BHA- pretreated groups were 3.5 and 4.7 times those of the control group at 2 h. The concentrations of the residual APAP in the BHA and BHT groups were almost 40 to 60% lower than those of the control group at 2 h, respectively, and the total excreted amounts of the residual APAP within 24 h were about 50 and 25% in the BHA and BHT groups, respectively. The concentration of APAP-sulfate in the BHT group was about 40% lower than that of the BHA group at 2 h but 1.5 and 1.3 times that at 6 and 8 h. Overall, these results indicate that BHA and BHT enhance the elimination of APAP from the blood with a concomitant increase in the appearance of APAP-glucuronide and APAP-sulfate in the blood.

4 Discussion

Numerous studies have indicated that the antioxidant BHA protects from an APAP-induced hepatotoxicity in laboratory animals. However, similar studies on the protective effect of the antioxidant BHT on the rats have been poorly reported. Previous studies by Haselton et al. demonstrated that BHA increased the glucuronidation capacity of APAP in vivo (16) and in vitro (23).
Therefore, in the present investigation, the effects of BHA and BHT on APAP metabolism by 7-day feeding have been examined in vivo. The results demonstrate that the excreted metabolite APAP-glucuronide in the urine of BHA-and BHT-fed groups was much higher than that of the APAP control group (Fig. 1). According to the time course (0-24 h) of the metabolites in the blood and urine, the protective effects of BHA and BHT require certain accumulation and stimulation in rats as xenobiotic substances to markedly change the metabolism of APAP.

Because APAP-glucuronide is the predominant metabolite formed after high doses of APAP, this pathway became the major focus of the biochemical studies. Table 1 shows the effect of BHA and BHT on hepatic UDP-GTase, the enzyme that catalyzes the conjugation of APAP with UDP-glucuronic acid and UDP-glucose dehydrogenase, an enzyme that plays a key role in UDP-glucuronic acid biosynthesis. An increase in the activity of UDP-GTase in BHA- and BHT-pretreated rats consequently enhances the pathway of acetaminophen glucuronidation in vivo. The present results are in agreement with those of several other investigations on the activities of the UDP-GTases in mice and rats pretreated with BHA- and BHT (24-26).

In addition to the action of BHA on the glucuronidation pathway, the antioxidant also affects glutathione metabolism. The results shown in Fig. 2 demonstrated that the excretions of APAP and its major metabolites in phase I were different between the BHA- and BHT-pretreated groups. Compared with BHA, BHT had a greater effect on the APAP-glucuronide excretion pathway and also on the APAP-sulfate excretion pathways. In our previous study (15) with diets containing BHA or BHT (0.5%) for 7 days, a significantly high concentration of GSH in the liver was witnessed in the BHA-APAP group, compared with that in the BHT-APAP and APAP control groups. We considered that BHA led to a much stronger accumulation of GSH in the rat liver than BHT did. The prevention of liver toxicity by BHA and BHT treatment may be explained by a decrease in NAPQI production and a subsequent decrease in covalent binding to cellular macromolecules. We reported that a clear difference had been proven for the function of BHT and BHA (17). The BHT group did not induce hepatic heat shock proteins 25 and 70i, while the BHA group did so in acetaminophen-induced hepatotoxicity as did the APAP control group.

These results suggest that different mechanisms may be involved in the effect of BHA and BHT against APAP-induced liver injury. The differences became apparent not only in phase II when NAPQI, an intermediate toxic metabolite of APAP, was oxidized by cytochrome P450, but also in phase I when the glucuronide and sulfate pathways existed.

5 Conclusions

BHA and BHT are chemically synthesized compounds. They are metabolized as xenobiotics in a living body through a biotransformation pathway. The rate of glucuronidation and sulfation of APAP was increased in rats fed with BHA or BHT. Both BHA and BHT have the same function to stimulate APAP-glucuronidation and APAP-sulfation that would play a major role in the acceleration of APAP excretion, however, BHT promoted the metabolism of APAP in phase I much more than did BHA. The function of this metabolic stimulation would protect the liver from APAP-induced hepatotoxicity as well. We confirmed that our previous presumption that BHA increases GSH accumulation in the liver and BHT induces a compound X (17). Both of them protect liver cells and maintain preventive functions against APAP-induced liver injury through detoxicant excretion resulting from conjugation with NAPQI.

References


