1 Introduction

Acetaminophen (APAP) is widely used as an over-the-counter drug for analgesic and antipyretic purposes. Although it is safe at therapeutic doses, accidental or intentional overdosing is frequent when APAP is taken in toxic amounts (1). Hepatotoxicity due to an APAP overdose has become a major problem and is a tremendous burden on the health care systems (2, 3). At normal dosage levels, APAP metabolizes through the major pathways that include glucuronidation (52%) and sulfation (42%) (4), but 4% of it is also oxidized to the highly reactive and toxic intermediate, N-acetyl-benzo-quinone-imine (NAPQI), by the cytochrome P450 (CYP) mixed function oxidases (5-7) and 2% of it is excreted as APAP alone in the urine. NAPQI has been shown to be detoxified by conjugation with glutathione (GSH) and eventually excreted in the urine in the form of a cysteine and mercapturic acid conjugate (5, 6, 8). When GSH levels are low, NAPQI fails to be completely detoxified by conjugation, and some of the unconjugated NAPQI covalently binds to critical cellular macromolecules (9, 10). The protein adduction that arises from this is thought to lead to liver injury, and

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Abstract: We assessed the protective effect of the dietary antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) against acetaminophen (APAP)-induced hepatotoxicity. After giving diets containing BHA (0.5%), BHT (0.5%) to male Wistar rats for 7 days, they were fasted for 16 h and then intraperitoneally injected with APAP (0.5g/kg). The severity of the hepatic injury was inferred from the plasma alanine aminotransferase and aspartate aminotransferase levels 24 h later. The results indicated that BHA and BHT significantly protected the rats against APAP hepatotoxicity. Upon investigating the mechanism of the protective action of BHA and BHT, the hepatic glutathione (GSH) levels decreased to the same degree in the APAP, BHA + APAP, and BHT + APAP groups 2 h after the injection of APAP, while the GSH levels started to recover in all groups at 6 h, but increasing faster in the BHA + APAP and BHT + APAP groups than in the APAP group. As compared with the control group, the hepatic cytochrome P450 (CYP) 2E1 levels tended to be about 20% higher in the BHA + APAP group from 2 to 24 h, and about 30% higher in the BHT + APAP group only at 24 h. These results suggest that the protection against APAP-induced hepatotoxicity provided by BHA and BHT is partly due to rapid recovery of the hepatic glutathione levels but does not involve changes in the CYP2E1 levels.

Key words: butylated hydroxyanisole, butylated hydroxytoluene, acetaminophen, glutathione, cytochrome P450, rat
ultimately to death in severe cases.

The formation of NAPQI depends on various forms of CYP. In rat liver microsomes, they have been identified as CYP1A1, 1A2 and 2E1 (11), and in mice as CYP1A2 and 2E1 (12). Studies conducted on CYP2E1 knockout mice showed that CYP2E1 is the most important factor in APAP bioactivation (12, 13), this providing evidence of the crucial role played by the CYP2E1 enzyme in the APAP-induced liver damage. The results of other studies suggest that APAP-induced liver injury is also caused by cellular oxidation stress, which leads to hepatic lipid peroxidation (14, 15).

Studies have so far conducted on butylated hydroxyanisole (BHA) (16) and on butylated hydroxytoluene (BHT) (17) for a protective effect against APAP-induced hepatotoxicity. However, no studies have been made to analyze the influence of BHA and BHT on GSH, CYP2E1 in the APAP-treated rats. We are now engaged in the study to compare such antioxidants between BHA and BHT on each action in the APAP-treated rats.

2 Materials and Methods

2.1 Chemicals

APAP was purchased from the Sigma Chemical Co. (St. Louis, MO) and the IATROZYME TA-LQ diagnostic kit was obtained from the Iatron Co. (IATRON Laboratories, Inc, Tokyo) for the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements. Rabbit anti-rat CYP 2E1 serum and rabbit anti-goat alkaline phosphatase-conjugated antibody obtained from Bio-Rad Co. (Bio-Rad Laboratories Co. Japan, Tokyo). BHA, BHT, and other chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka).

2.2 Animals and Experimental Design

Five-week-old male Wistar-SPF rats (SLC Co., Japan) were individually kept under a 12h light/dark cycle. 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) for three days, and then to a standard diet (AIN-76 rodent diet) for four days. The composition of the standard diet is as follows (g/100g): Casein 20, Cornstarch 40, Sucrose 25, Corn oil 5, AIN76™ Vitamin 1, AIN76™ Mineral 3.5, Cellulose 5, Choline 0.2, Methionine 0.3. The rats were then divided into four groups. One each of two groups was given an experimental diet containing BHA (0.5%) or BHT (0.5%) and the other two groups received the standard diet only for seven days. For the experimental diet, total amount of cornstarch and antioxidants were fixed at 40g/100g of diet. Three of these four groups (except one standard diet group) were then treated with APAP.

The rats were fasted for 16 h before intraperitoneal administering APAP at a dose of 500 mg/kg per body weight and the control group received the same volume of saline instead of APAP. At 6 h following the APAP dosing, the rats were put back on the respective diets which they had previously received and six rats in each diet groups were killed by decapitation at the indicated time. Blood and liver samples were collected and analyzed as follows.

2.3 ALT and AST Activities of Plasma

The ALT and AST levels in the plasma were determined using the commercial kit according to the manufacturer's instructions.

2.4 Measurement of GSH and GSSG Levels in Rat Livers

The liver samples (0.5 g) were homogenized with 5ml of 7% perchloric acid solution containing 2mM phenanthroline on ice for 10 min, and then centrifuged at 15,000 rpm for 10 min at 4°C. The GSH and glutathione disulfide (GSSG) concentrations in the supernatants were then measured by HPLC according to the method of Reed (18) using 20 µl samples that were injected into the HPLC instrument (L-7100 intelligent pump with UV detector (Hitachi, Ltd., Tokyo). A Waters µBondapak™ NH2 column (3.9 × 300 mm; Particle size 10 µm, Waters Chromatography Division/Milipore, Milford MA) was attached to the HPLC instrument via a guard column. The analytical conditions were as follows: UV-detection, 365 nm; column temperature, 30°C; flow rate, 1.0 ml/min; mobile phase, according to the method of Reed (18).

2.5 Microsome Preparation and Western Immunoblots of CYP 2E1 Protein

Microsomal pellets were obtained from the liver homogenates (25% wt/vol in 1.15% KCl-0.01 mol phosphate buffer, pH7.4 plus 1mM phenyl methyl sul-
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fonyl fluoride) by centrifugation at 105,000 × g at 4°C for 60 min according to the method of Wang (19). The protein content was measured according to the method of Lowry (20).

The microsomal proteins were electrophoretically separated on a 10% SDS polyacrylamide gel and the CYP isozyme-containing proteins were transferred to a nitrocellulose membrane by the method of Towbin (21) and exposed to a polyclonal antibody (rabbit anti-rat CYP 2E1 serum) solution at a 1:1000 dilution in PBS containing 0.5% BSA, then incubated at 37°C for 2 h with continuous shaking. After washing, the membrane was incubated with a rabbit anti-goat alkaline phosphatase-conjugated antibody at a 1:10000 dilution in TBS (50mM Tris, 200mM NaCl) for 1 h at 37°C with continuous shaking. The immunoreactivity was visualized using the Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences Japan Co., Ltd., Tokyo), and the membrane scanned with a Personal Laser Densitometer. The data were quantified using an image gauge.

2.6 Statistical Analysis

The data are shown as the means ± SE (N = 6). The 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.

3 Results and Discussion

3.1 Effect of BHA and BHT on APAP-induced Toxicity

No significant differences were observed in weight gain or feed efficiency among the groups of rats over the 7-day period while they received the experimental diets. The amount of APAP used in the present study was greater than that used in the usual medical objectives. We set up the amount probability of APAP administration at 500, 600, 700 mg/kg, and investigated the survival probability of the rats. The AST activities of the plasma were 2317, 2658 and 3076 IU/L, respectively. The survival ratios at 24 h after the APAP administration at 500 mg/kg, 600 mg/kg and 700 mg/kg were 100%, 63% and 41%, respectively. Figure 1 shows the plasma AST and ALT levels at 24 h after the APAP administration. While there were almost the same AST levels in all groups during the first 8 h following the APAP administration, the significant increase of the AST was observed only in the APAP group at 24 h.

BHA + APAP and BHT + APAP groups were 10 to 15% higher than those of the control groups which means about 90 to 85% lower levels than that of the APAP groups, respectively. The results showed that BHA and BHT were effective in protecting rats from the hepatotoxicity of APAP, as assessed through the suppression of the plasma AST and ALT levels.

Figure 2 shows the change in the plasma AST levels from 0 to 24 h following the administration of APAP. The AST levels in all groups during the first 8 h following the APAP administration, the significant increase of the AST was observed only in the APAP group at 24 h.

3.2 Effect of BHA and BHT on Hepatic GSH and GSSG Levels

Figure 3 shows the effect of the BHA and BHT pretreatments on the hepatic GSH levels following the APAP administration. The maximum depletion in the GSH levels occurred at 2 h after the administration of APAP and the levels in all groups were significantly lower than that of the control group at 0 h (P < 0.01). Differences in the GSH levels among the APAP treated groups up to 4 h after the administration of APAP were not significant, however, the GSH levels started to recover in the BHA and BHT pretreated groups at this point. The results showed that both antioxidants accelerated the recovery of the GSH level after the maximum depletion had occurred. The GSH levels of all groups were increased after 6 h (APAP, 51%; BHA + APAP, 56%; BHT + APAP, 58%).
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123%; BHT + APAP, 100% against the control group at 0 h), and that of the BHA + APAP group was significantly higher at 24 h (27 ± 1.83 μmol/g, 171%, P < 0.01) than that of the control group.

APAP bioactivation is the first step in the initiation of the APAP hepatotoxicity. Depletion of the hepatocellular GSH is the critical sign of the APAP bioactivation and leads to the production of a highly reactive interme-
diate, NAPQI. It was shown that GSH was depleted to more than 80% at 2 h after APAP injection regardless of the pretreatment with BHA and BHT. This observation is consistent with the findings from recent studies in which GSH was depleted by APAP to a similar extent (31, 32), however, the GSH levels of our experiment with BHA and BHT recovered more rapidly. It was unlikely that the protective effects of BHA and BHT are due to reduced bioactivation of APAP, since these substances have little influence on the GSH levels in the first 2 h of the GSH-monitoring. The fact that BHA was very effective in raising the GSH levels during the latter period of the experiment (6-24 h) suggests that BHA played a greater role in inhibiting the progression of toxic injury than in preventing initiation of the toxicity. Studies conducted hitherto prove that BHA increases the GSH levels in the liver and kidneys of female mice (33, 34).

The change in the total hepatic glutathione (GSH + GSSG, glutathione disulfide) levels from 0 to 24 h following APAP treatment was a trend similar to those of GSH (data not shown). The GSH levels of the control group also markedly increased at 24 h (about 3 times versus 0 h). This suggests that there had been a "rebound" in the GSH levels due to the refeeding following 22 hours of fasting.

![Fig. 2](image_url)  
**Fig. 2** Effect of BHA or BHT Pretreatment on APAP-administered Rats. Time courses after APAP administration of plasma AST activities of six rats in the following each groups; control (no treatment, no administration), ○: APAP, ■: BHA + APAP, ▲: BHT + APAP, △. Each point represents the means ± SE **P < 0.01 when compared to the control level.

![Fig. 3](image_url)  
**Fig. 3** Effect of BHA or BHT Pretreatment on Hepatic GSH Contents of APAP-administered Rats. GSH of the control group without APAP administration (○) was measured at time 0, 8 and 24h. GSH contents of APAP (■), BHA + APAP (▲), and BHT + APAP (△) groups were measured at 0 (before APAP administration), 2, 4, 6, 8, and 24h after APAP administration. Each point represents the mean ± SE of 6 rats. **P < 0.01, *P < 0.05 when compared to the control level.
3.3 Effect of BHA and BHT on CYP2E1 Contents

We estimated the liver concentrations of CYP2E1 by Western immunoblotting to determine whether this protein is involved in the protective action of BHA and BHT against APAP-induced hepatic injury. **Figure 4** (a) shows the Western immunoblotting patterns of CYP2E1 after 0-24 h of APAP administration following the BHA or BHT pretreatment. **Figure 4** (b) shows the relative contents (%) of CYP2E1. The BHA + APAP group showed higher contents up to 8 h, significantly at 2 and 4 h, while the BHT + APAP group showed significantly higher contents at 24 h. CYP2E1 is the key enzyme for the bioactivation of APAP. In previous research, it was found that treatment with retinol had no effect on CYP2E1 (22) and that CYP2E1 is not essential for a response to short-term treatment with alcohols (23), but hepatic CYP2E1 activity was enhanced in NDN (non-diabetic patients with non-alcoholic steatohepatitis) (24). Recent studies accepted the increase in the CYP concentration due to the fasting for rats. Since the fasting was initiated, glycogen decreased and glucuronidation was prevented. Consequently the CYP activities increased. We have confirmed that the CYP2E1 concentration of APAP in three treated groups increased 10-30% at 24 h. These increases are considered to be caused by the fasting.

Although experimental evidence suggests that CYP2E1 is the most important CYP isoform in the APAP-induced hepatotoxicity (25, 26), it has been reported that the CYP1A1 and CYP1A2 forms are also involved in the metabolic activation of APAP and its

![Fig. 4](image-url)
toxicity in rats (27-30). As CYP2E1 was not suppressed by BHA and BHT, the hepatotoxicity seemed to be suppressed by the BHA pretreatment with an increase in the GSH content. On the other hand the detoxification of APAP by BHT must have been performed through a different route from BHA. Thus, a further study of their action mechanism is needed to be more precisely determine, id est the stage(s) of the detoxification process, whether or not their effect is manifested, and whether or not the enzymes are actually involved.

While CYP2E1 activities increased on the one hand, the GSH reservoir decreased on the other at 2 h and 4 h. BHA pushed up the GSH levels of rats and therefore its mechanism against the APAP liver injury considered to be different from the BHT’s.

4 Conclusion

We found that BHA and BHT have a protective effect against the APAP-induced hepatotoxicity. It is considered that the BHA and BHT are likely to be effective as they are chemically synthesized products and xenobiotic to the living body. The experiment conducted in 0-24 h showed a quick recovery of the GSH levels in the group with BHA. Although the recovery of the GSH-level of BHT + APAP group was not as rapid as that in the BHA-APAP group, the recovery was faster than the APAP group. Presumably, BHA and BHT have some influence on the output volume of NAPQI, an intermediate product of APAP, because the recovery of GSH should be slow when the NAPQI output volume is large.

The molecular basis of the APAP toxicity remains largely unknown, and although knowing the proteins involved in covalent binding (35) does help somewhat, our understanding of this phenomenon is far from complete. The suggested mechanisms for the inhibitory effects of BHA and BHT on the APAP-induced hepatotoxicity include the following: (1) Direct effects of BHA and BHT on APAP metabolism, (2) Competitive inhibition of CYP-mediated APAP metabolism by BHA and BHT, (3) Interaction of oxidative stress-derived metabolites with proteins critical to the toxicity, and (4) Influence of BHA and BHT on the immunity system. The possible involvement of such events in the mechanisms should be explored at the biochemical and molecular levels in the future.

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