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Effects of BHA and BHT on Bromobenzene-induced Hepatotoxicity.

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〈**Abstract**〉

Effects of BHA and BHT on bromobenzene-induced hepatotoxicity were studied. BHA and BHT were given to rat in the diet at 0.25% level for 10 days prior to bromobenzene challenge. Pretreatment with BHA retrieved the bromobenzene-incluced toxicity: i.e. recovered the reduced biliary excretion of BSP and the enhanced SGPT activity to control levels. However, BHT treatment resulted in a severe necrosis and further decreased the biliary excretion of BSP. Addition of bromobenzene to hepatocyte culture weakly induced the malondialdehyde (MDA) production and the addition of BHA and BHT did not protect against the enhanced MDA production.

Bromobenzene에 의한 간독성에 대한 BHA와 BHT의 영향

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〈**요 약**〉

식품에 항산화제로 첨가하는 BHA와 BHT의 bromobenzene에 의해 유발되는 간독성에 대한 영향을 조사하였다. BHA와 BHT는 bromobenzene을 처리하기 전 10일간 0.25%로 사료에 첨가하였다. BHA는 bromobenzene에 의해 유발되는 BSP의 담즙을 통한 배설의 억제현상과 담즙배설 억제작용, 그리고 SGPT 활성의 상승현상 모두에 대해 보호작용을 나타내었다.

그러나 BHT는 위의 모든 현상에 대해 보호작용을 나타내지 못하고 오히려 더욱 악화시켰다. 또한 이들 항산화제는 *in vitro* 상태에서 malondialdehyde(MDA)의 증가에 대해 보호작용을 나타내지 못하였다.

I. Introduction

Intraperitoneal administration of bromobenzene to rats results in a rapid and extensive depletion of glutathione from the liver[9,10] and subsequently leads to the centrilobular necrosis of the liver.

The hepatic biotransformation of bromobenzene to chemically reactive 3,4-bromobenzene oxide by cytochrome p-450 dependent mixed function oxidases is apparently responsible for its hepatotoxicity in the rat[8,16], the latter being correlated with covalent binding of this metabolite to liver macromolecules.[17] This phenomenon occurs when dose and/or rate of biotransformation of bromobenzene exceed the capacity of the liver to inactivate the epoxide,

the latter being dependent on microsomal epoxide hydrase and, more importantly, on glutathione availability for conjugation of the epoxide by cytosolic glutathione S-transferase.[10,20,22]

Enhancement of the rate of biotransformation of bromobenzene to 3,4-bromobenzene oxide by phenobarbital pretreatment increases bromobenzene liver toxicity.[4] Induction of liver microsomal mixed-function oxidases by 3 methylcholanthrene, on the other hand, does not increase hepatic necrosis after bromobenzene even though metabolism of the latter is also significantly enhanced.[22] This inducer favors biotransformation of bromobenzene to 2,3-bromobenzene oxide which is apparently non toxic[22], probably because of its lower reactivity and different intracellular covalent binding sites.[13] Recent studies moreover tend to confirm the existance of a close correlation among *in vivo* hepatic necrosis, rate of disappearance of p-bromophenol via 3,4 epoxidation of bromobenzene as opposed to formation of o-bromophenol via 2,3-epoxidation[22].

Administration of BHT or BHA induces a particular form of liver cytochrome P-450 and increases the activity of several microsomal mixed function oxidases.[6] This increase could thus lead to potentiation of bromobenzene hepatotoxicity by these antioxidants. Treatment with these antioxidants also increases liver epoxide hydrase and glutathione S-transferase as well as liver glutathione content.[1,6,15]

I thus examined the effect of these antioxidants on hepatic function, as reflected by biliary excretion of BSP, which should be conjugated with glutathione before excreted into bile, and examined the effect on liver necrosis as reflected by transaminase activity.

Ⅱ. Materials and Method

Animals; Male Sprague-Dawley rats(200 -250g) were used. Rats were maintained on a powdered diet(Purina Korea) and water was given *ad libitum*. The animals were kept in 12 hr light/dark cycle.

Biliary excretion of BSP: Biliary excretion of BSP was determined as described previously.[7] BSP(40mg/kg) was injected iv 24hr after bromobenzene (2.5mmole/kg, ip) and bile was collected for 30min. BHT and BHA were given to rat in the diet at 0.25% level for 10 days prior to bromobenzene challenge. The weight of bile in each samples was determined gravimetrically assuming a specific gravity of 1.0.

SGPT activity determination: SGPT activity was determined colorimetrically using transaminase Kit No. 55-UV(Sigma).

Primary rat liver cell culture: Hepatocytes were isolated from rats by a collagenase perfusion technique and as monolayers in serum-free medium on 60mm collagen-coated plates as described previously.[21]

In vitro lipid peroxidation: Four hours after initial plating, the cultrue medium was changed to fresh medium and bromobenzene, BHT and BHA were dissolved in dimethyl sulfoxide (DMSO) and added to the culture at the concentrations indicated. The final concentration of DMSO in the culture was 0.1%(V/V). The culture medium was removed for determination of the malonaldehyde 6hr after treatment.[18]

Statistics: Results were expressed as the mean ±SE. The siginificance of the difference between mean values was assessed by Student's t-test ($P < 0.05$).

Ⅲ. Results

As indicated in Figure 1 and Table 1,

Table 1. Effects of BHA and BHT on bromobenzene-induced hepatotoxicity[a]

Bromobenzene (m mole/kg)	Treatment[b]	% Liver	Bile flow (μl/min/g)	BSP (% dose/30min)	SGPT (units/ml)
0	—	2.89±0.06	1.98±0.08	54.9±1.00	34.2±3.0
2.5	—	3.15±0.09	2.19±0.27	40.6±1.05[c]	86.5±5.6[c]
2.5	BHT	5.55±0.12[d]	1.03±0.07[d]	23.6±1.28[d]	NA[e]
2.5	BHA	3.46±0.03[d]	2.64±0.19[d]	57.8±2.01[d]	38.5±0.8[d]

a. Results for each group are given as mean±SE for 4 rats.
b. Rats were fed diets containing 0.25% BHT or BHA for 10 days prior to the ip administration of bromobenzene.
c. Significantly difference ($p < 0.05$) between the control group and the bromobenzene-treated group.
d. Significantly difference ($p < 0.05$) between the bromobenzene-treated group and the antioxidant-treated group.
e. No activity.

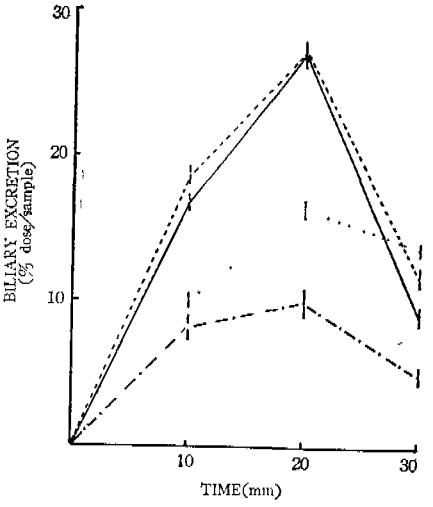

Fig. 1. Effect of antioxidants on bromobenzene-induced depression in the biliary excretion of BSP.
Rats were fed diets containing 0.25% BHA or BHT prior to the ip administration of bromobenzene(2.5 m mol/kg). The experiments were performed 24hr postinjection.

Treatment groups are depicted as control ( ), bromobenzene( ), BHA plus bromobenzene(···· ···), and BHT plus bromobenzene(-·-· ·). The vertical line at each point represents the mean±S.E of 4 rats.

treatment of bromobenzene significantly delayed the biliary excretion of BSP into bile in the rats and increased in SGPT activity.

Pretreatment with BHT resulted in a severe necrosis and hepatomegaly and further decreased the biliary excretion of BSP. The bile flow was also decreased in the BHT-treated rats. So pretreatment with BHT exacerbated the bromobenzene-induced hepatotoxicity. No activity of SGPT however was shown in the BHT-treated rats. The plausible reason for such result was not clarified.

Effects of BHA-treatment was quite different from that of BHT. Pretreatment with BHA retrieved the bromobenzene-induced toxicity in the above parameters: i.e. recovered the reduced biliary excretion of BSP and enhanced SGPT activity to control levels.

To further investigate the effect of these antioxidants on bromobenzene-induced hepatotoxicity, the malondialdehyde (MDA) production was assessed in hepatocyte culture systems. Addition of bromobenzene to cultures weakly induced the MDA production and the addition of BHT did not protect against the enhanced MDA production(Table 2). These results in hepatocyte cultures suggested that lipid peroxidation may have not an early and critical role in bringing about the bromobenzene-induced hepatotoxicity. It therefore concluded that the lipid peroxidation which occured during bromobenzene toxicity was merely a consequence of glutathione (GSH) depletion and cell death.[19]

Table 2. Effect of BHT and BHA on the bromobenzene-induced malondialdehyde production in primary cultures of adult rat hepatocytes[a]

Treatment	Malondialdehyde (nmole/mg protein)
DMSO control	0.19±0.01[b]
Bromobenzene(0.5mM)	0.30±0.03[c]
Bromobenzene+BHT(100μM)	0.29±0.04[c]
Bromobenzene+BHA(100μM)	0.30±0.03[c]

a. After incubation for 4hr to allow the attachment of viable cells, bromobenzene, BHT and BHA were dissolved in dimethyl sulfoxide (DMSO) and added to the cultures at the concentrations indicated. The culture medium was removed for determination of the malondialdehyde 6hr after treatment.
b. Results are the mean±SE of the determinations on 3 separate cultures.
c. Significantly different from controls ($p < 0.05$).

Ⅳ. Discussion

This studies demonstrated that dietary BHA prevents or alleviates the acute toxicity induced by bromobenzene in rats as judged by SGPT activity and biliary excretory function. Recently, Miranda et al.[14] reported that prior consumption of a diet containing BHA by female mice prevented the development of or minimized the acute liver damage caused by bromobenzene, which was judged by plasma GPT and GOT levels, hepatic cytochrome p-450 content, and liver histology.

Bromobenzene is metabolized to reactive intermediate(s) which are inactivated by conjugation with GSH. Increased cell injury is observed with GSH-depleted livers which, in turn, are protected by addition of GSH or facilitated GSH synthesis.[18,20]

Several mechanisms might be postulated to explain the protective action of BHA. A possible mechanism for protection could be ascribed to the ability of BHA to reduce the formation of reactive metabolites of bromobenzene such as the conversion to toxic 3, 4-bromobenzene oxide.

Pretreatment with BHA increases in liver GSH[2,11,14], and this nucleophile can bind the toxic metabolite of bromobenzene, decreasing the availability of reactive metabolite, and consequently reducing with more critical sites in hepatocytes.

In addition, the activity of detoxifying enzymes such as epoxide hydrase[6,11], more importantly, glutathione S-transferase[3,11] is enhanced by BHA in rodents.

As an antioxidant, BHA may also react directly with free radicals and other toxic reactive intermediates generated in vivo.

Pretreatment with BHT however exacerbated the bromobenzene-induced hepatotoxicity. The biliary excretion of BSP and the bile flow was extensively decreased in the BHT-treated rat compared to the bromobenzene-treated rats. Similar to BHA, when rats were treated with BHT, glutathione S-transferase activity in the liver increased several folds.[1] The deteriorative action of BHT therefore appear to be due mainly to enhancing the formation of toxic bromobenzene oxide. The plausible reason for no activity of SGPT shown in the BHT-treated rats was not clarified.

For bromobenzene toxicity, the importance of peroxidation vs. arylation is a matter of continued debate and controversy. Recently, Casini et al.[5] demonstrated that lipid peroxidation is an early important event in the toxicity of bromobenzene to monolayer cultures of rat hepatocytes. The major piece of evidence which supports this conclusion is the finding that the antioxidants, DPPD, BHA, and BHT, delays, but does not prevent the toxicity of bromobenzene to cultured hepatocytes.

Smith et al.[19] however studied the relationship between the time of onset of lipid peroxidation and cell viability in exposed to either CCl_4 or bromobenzene, and concluded that the lipid peroxidation which occurred

during bromobenzene toxicity was merely a consequence of GSH depletion and cell death.

Unlike the previous report[5], the addition of BHT or BHA did not reduced the bromobenzene-induced increase in MDA production. In addition, the addition of the same concentration of BHT or BHA significantly reduced the CCl_4-induced increase in MDA production.[7]

From above *in vivo* and *in vitro* results, it therefore is concluded that lipid peroxidation occurs at a later stage and only after cell death in bromobenzene cytotoxicity.

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References

1. Awasthi, Y.C., Partridge, C.A., and Dao, D.D. Effect of butylated hydroxytoluene on glutathione S-transferase and glutathione peroxidase activities in rat liver. Biochem. Pharmacol., 32 : 1197—1200(1983)
2. Batzinger, R.P., Ou, S. -Y. L., and Bueding, E. Anti-mutagenic effects of 2(3)-tert-butyl-hydroxy-anisole and of microbial agents. Cancer Res., 38 : 4476—4483(1978).
3. Benson, A.M., Batzinger, R.P., Ou, S. -Y. L., Bueding, E., Cha, Y. -N., and Talalay, P. Elevation of hepatic glutathione S-trasferase activities and protection against mutagenic metabolites of benzo(a)-pyrene by dietary antioxidants. Cancer Res., 38 : 4486—4495(1978).
4. Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G., and Gillette, J.R. Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc. Nat. Acad. Sci. USA, 68 : 160—164(1971).
5. Casini, A., Giorli, M., Hyland, R.J., Serroni, A., Gilfor, D., and Farber, J.L. Mechanism of cell injury in the killing of cultured hepatocytes by bromobenzen. J. Biol. Chem., 257 : 6721—6728(1982).
6. Cha, Y.-N., Martz, F., and Bueding, E. Enhancement of liver microsome epoxide hydratase activity in rondets by treatment with 2(3)-tert-butyl-4-hydroxyanisole. Cancer Res., 38 : 4494—4496(1978).
7. Choe, S.Y., and Yang, K.H. Effect of butylated hydroxytoluene(BHT) and butylated hydroxyanisole (BHA) on carbon tetrachloride and TCDD hepatotoxicity. Korean Biochem. Journal, 16 : 315—321(1983).
8. Gillette, J.R. A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity. II. Alterations in the kinetics of covalent binding. Biochem. Pharmacol., 23 : 2927—2938(1974).
9. Hetu, C., Dumont, A., and Joly, J. -G. Effect of chronic ethanol administration on bromobenzene liver toxicity in the rat. Toxicol. Appl. Pharmacol., 67 : 67 : 166—177 (1983).
10. Jollow, D.J., Mitchell, J.R., Zampaglione, N., and Gillette, J.R. Bromobenzene-induced liver nocrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology, 11 : 151—169(1974).
11. Lam, L.K.T., Sparnins, V.L., Hochalter, J.B., and Wattenberg, L.W. Effects of 2- and 3-tert-butyl-4-hydroxyanisole on glutathione S-transferase and epoxide hydrolase activities and sulfhydryl levels in liver and forestomach of mice. Cancer Res., 41 : 3940—3943(1981).
12. Lau, S.S., Abrans, G.D., and Zannoni, V.G. Metabolic activation and detoxification of bromobenzene leading to cytotoxicity. J. Pharmacol. Exp. Ther., 214 : 703—708(1980).
13. Lau, S.S., and Zannoni, V.G. Bromobenzene epoxidation leading to binding on macromolecular protein sites. J. Pharmacol.

Exp. Ther., 219 : 563—572(1981).

14. Miranda, C.L., Henderson, M. C., Schmitz, J.A., and Buhler, D.R. Protective role of dietary butylated hydroxyanisole against chemical-induced acute liver damage in mice. Toxicol. Appl. Pharmacol., 69 : 73—80(1983).
15. Miranda, C.L., Reed, R.L., Cheeke, P. R., and Buhler, D.R. Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. Toxicol. Appl. Pharmacol., 59 : 424--430(1981).
16. Mitchell, J.R., Reid, W.D., Christie, B., Moskowitz, J., Krishna, G., and Brodie, B. B. Bromobenzene-induced hepatic necrosis: Species differences and protection by SKF 525-A. Res. Commun. Chem. Pathol. Pharmacol., 2 : 877(1971).
17. Reid, W.D., and Krishna, G. Centrilobular hepatic necrosis related to covalent binding of metabolites of halogenated aromatic hydrocarbons. Exp. Mol. Pathol. 18 : 80—99(1973).
18. Smith, M.T., Thor, H., Hartzell, P., and Orrenius, S. The measurement of lipid peroxidation in isolated hepatocytes. Biochem. Pharmacol., 31 : 19—26(1982).
19. Smith, M.T., Thor, H., and Orrenius, S. The role of lipid peroxidation in the toxicity of foreign compounds to liver cells. Biochem. Pharmacol., 32 : 763—764(1983).
20. Thor, H., Moldeus, P., Kristoferson, A., Hogberg, J., Reed, D.J., and Orrenius, S. Metabolic activation and hepatotoxicity. Metabolism of bromobenzene in isolated hepatocytes. Arch. Biochem. Biophys., 188 : 114—121(1978).
21. Yang, K.H., Choi, E.J., and Choe, S.Y. Cytotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin on primary cultures of adult rat hepatocytes. Arch. Environ. Contam. Toxicol., 12 : 183—188(1983).
22. Zampaglione, N., Jollow, D.J., Mitchell, J.R., Stripp, B., Hamrich, M., and Gillette, J.R. Role of detoxifying enzymes in bromobenzene-induced liver necrosis. J. Pharmacol. Exp. Ther., 187 : 218—227(1973).