Korean J. Toxicol.
Vol. 11, No. 2, pp. 215-221, 1995

Long-Term Feeding of Dietary Fat and Butylated Hydroxytoluene on The Hepatic Microsomal Mixed-Function Oxidase System in 2-Acetylaminofluorene Treated Rats

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(Received November 16, 1995)
(Accepted November 25, 1995)

ABSTRACT : *This paper examines the effects of dietary polyunsaturated fatty acid/saturated fatty acid (p/s) ratios and butylated hydroxytoluene (BHT) on the hepatic microsomal mixed-function oxidase system in 2-acetylaminofluorene (2-AAF) treated rats. Sprague-Dawley male rats were fed the diet of beef tallow (p/s 0.08), beef tallow plus soybean oil (p/s 1.0), and soybean oil (p/s 4.0) at the level of 15% fat and with or without 0.3% BHT. After 2-AAF was injected twice at the ages of 23 and 27 weeks, cholesterol/phospholipid molar ratio, thiobarbituric acid reactive substances (TBARS) level, cytochrome P-450, cytochrome b_5, NADPH-cytochrome b_5, and NADPH-cytochrome c reductase activity were measured from isolated hepatic microsomal fractions. In the beef tallow (p/s 0.08) and beef tallow plus soybean oil (p/s 1.0) groups, cholesterol/phospholipid molar ratio showed decreasing tendency by 2-AAF and BHT. Cytochrome P-450 content was decreased in the group of soybean oil (p/s 4.0) and NADPH-cytochrome c reductase activity was increased by 2-AAF and BHT in all the dietary groups. While TBARS levels were increased by 2-AAF in all the dietary groups, they were reduced by BHT in the soybean oil (p/s 4.0) group. These results suggest that long term intake of soybean oil (p/s 4.0) diet induced changes in the nature of microsomal membrane and induced less cytochrome P-450, low level feeding of BHT increased cytochrome c reductase activity and lowered microsomal lipid peroxidation levels, which were increased by 2-AAF treatment.*

Key Words : *Butylated hydroxytoluene, Dietary fatty acids, Hepatocarcinogenesis, Mixed-function oxidase system*

I. INTRODUCTION

Several epidemiological studies showed that there are some relationships between consumption of dietary fat and prevalence of cancer (Zaridze *et al.*, 1985; Wynder *et al.*, 1976). According to animal studies, it has been consistently shown that certain types of cancer develop more readily in animals fed high-fat diets compared to those fed low-fat ones (Rose *et al.*, 1985). From some experiments with laboratory animals, it was also conjectured that polyunsaturated fatty acids (PUFA) are more effective for carcinogenesis than saturated fatty acids (Hopkins and Carrol, 1979; Roebuck *et al.*, 1981). The mechanism involved in the promoting action of PUFA is not known yet, although it has been conjectured that those acids might provide favorable conditions for tumor growth.

It is well known that a large number of foreign and endogenous compounds are metabolized in mammals by a mixed-function oxidase (MFO) system localized in the microsomal membranes of the hepatocytes (Remmer, 1970; Coon, 1978; Robert *et al.*, 1993), which consists of cyotchrome P-450, cytochrome c reductase, and cytochrome b_5. Phospholipids is also required for proper function of enzymes bound to these membranes (Lu, 1976). Marshall (1971) and Norred (1972) have shown that liver microsomes from rats fed diets containing increased amounts of corn oil contain increased amounts of cytochrome P-450. However, another study indicated that the concentration of hepatic microsomal cytochrome P-450 is decreased when rats are fed a diet containing PUFA rather than saturated ones (Hopkins and West, 1976).

Butylated hydroxytoluene (BHT), an antioxidant commonly used as a food additive, reduced the incidence of tumorigenesis when fed simutaneously with the carcinogen to rats (Wattenberg, 1978). The use of antioxidants as possible inhibitors of the chemical carcinogens has been based in general on the concept that the antioxidants may protect cellular constituents from attack by exerting a scavenging effect on the reactive species of carcinogens. BHT might change metabolism rates of carcinogens as well. A number of studies have demonstrated that it is possible to protect cellular constituents against chemical carcinogens by administrating inducers which increase MFO system activities (Maikiura *et al.*, 1974).

In this experiment, we have examined the influences of dietary fats and BHT on the activities of several enzymes in hepatic microsomal MFO system. cholesterol/phospholipid molar ratio, thiobarbituric acid reactive substances (TBARS) level, concentration of cytochrome P-450, cytochrome b_5, and NADPH-cytochrome c reductase activity determined from isolated hepatic microsomes in male rats.

Table 1. Composition of experimental diet (g/100 g diet)

Ingredient	Beef tallow diet (p/s 0.08)	BT+SOY[1] (p/s 1.0)	Soybean oil diet (p/s 4.0)
Corn starch	54.7	54.7	54.7
Casein	20.0	20.0	20.0
Cellulose	5.0	5.0	5.0
Soybean oil	-	7.5	15.0
Beef tallow	15.0	7.5	-
Vitamin mixture[2]	1.0	1.0	1.0
Salt mixture[3]	4.0	4.0	4.0
DL-Methionine	0.3	0.3	0.3

[1]BT+SOY: Beef tallow + Soybean oil diet.

[2]Nutritional Biochemicals, ICN Life Science Group, Cleveland, Ohio.Vitamin mixture is composed of; Vit.A Acetate (500,000 IU/g) 1.8 g, Vit.D conc. (850,000 IU/g) 0.125 g, γ-Tocopherol (250 IU/g) 22.0 g, Ascorbic acid 45.0 g, Inositol 5.9 g, Choline chloride 75.0 g, Menadione 2.25 g, p-Aminobenzoic acid 5.0 g, Niacin 4.25 g, Riboflavin 1.0 g, Pyridoxine hydrochloride 1.0 g, Calcium pantothenic acid 3.0 g, Biotin 0.02 g, Folic acid 0.09 g, Vitamin B_{12} 0.00135 g, and Dextrose to 1 kg.

[3]Composition of Salt mixture, g/kg mixture: $CaHPO_4$ 500 g, NaCl 74 g, K_2SO_4 52 g, Potassium citrate monohydrate 22 g, MgO 24 g, Manganase Carbonate (43~48% Mn) 3.5 g, Ferric Citrate (16~17% Fe) 6.0 g, Zinc Carbonate 1.6 g, Cupric Carbonate (53~55% Cu) 0.3 g, KIO_3 0.01 g, Chromium Potassium Sulfate 0.55 g, $Na_2SeO_3 \cdot 5H_2O$ 0.91 g, Sucrose, finely powered 118.0 g.

II. MATERIALS AND METHODS

1. Animals, Diets and Chemicals

Male Sprague-Dawley rats weighing 50-60 g were obtained from Experimental Animal Laboratory at Seoul National University. All animals were fed diet and water ad libitum throughout the entire experiment period. Experimental diet contained 15% (w/w) fat consisting of beef tallow, beef tallow plus soybean oil, and soybean oil with and without BHT (0.3% of diet). At the ages of 23 and 27 weeks, rats were injected 2-acetylaminofluorene (2-AAF, 50 mg/Kg body weight). The composition of diet and experimental design are given in Table 1 and Fig. 1, respectively.

2. Preparation of Hepatic Microsomes

Animals were killed by decapitation, and livers were quickly excised, rinsed with ice-cold physiological saline, blotted, weighed, and minced. Livers were homogenized in 150 mM KCl, 10 mM phosphate buffer (pH 7.4) with tissue homogenizer. Homogenates were centrifuged at 12,000×g for 20 min. The microsomal pellet was obtained by centrifugation of the 105,000×g for 60 min. The microsomal pellets were stored at -40°C until assayed.

3. Biochemical Analysis

Microsomal lipids were isolated by Folch (1957) extraction, and inorganic phosphorus was determined by the method of Bartlett (1959). The cholesterol contents of the microsomal lipids was determined using the method of Searcy *et al* (1969). Microsomal lipid peroxidation was estimated by measuring the amount of thiobarbituric acid reactive substances (TBARS) from malondialdehyde (MDA) concentration (Bidlack and Tappel, 1973). The cytochrome P-450 content was determined by differential CO-binding spectra using dithionite as the reducing agent, according to the method of Omura and Sato (1964). The concentration of the heme proteins were expressed as nmoles·mg protein^{-1}. NADPH-cytochrome c reductase activity was determined by measuring the rate of disappearance of oxidized dichlorophenol-

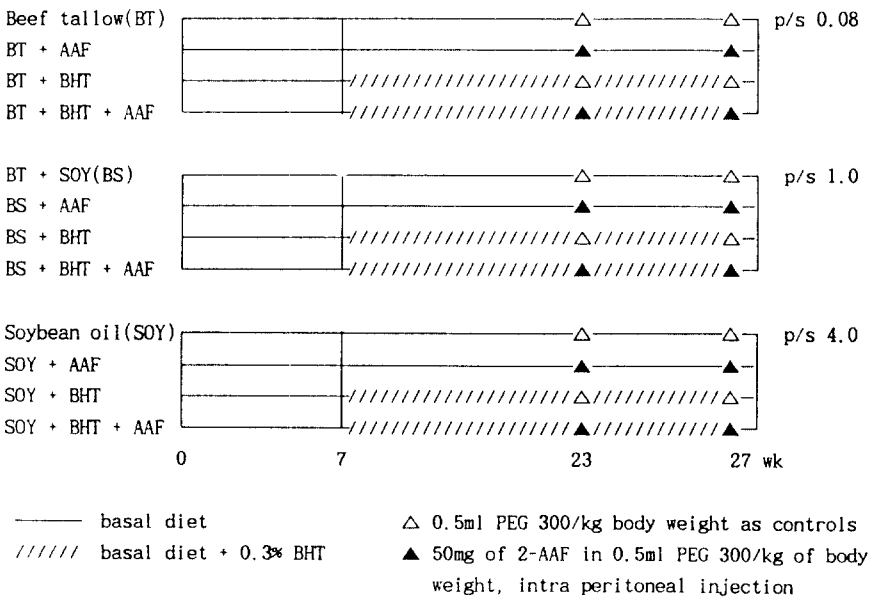


Fig. 1. Scheme of experimental design.

indophenol (DCIP) at 600 nm (Master *et al.*, 1967). The reaction was run at 30°C and pH 7.7 with 1 to 2 mg of microsomal protein, 96×10^{-9} moles DICP and 10^{-3} M NADPH in the mixture. The reaction was initiated by the addition of the NADPH, and the enzyme activity was expressed as nmole·min^{-1}mg $protein^{-1}$. Total microsomal protein concentrations were measured using the method of Lowry (1951) and using bovine serum albumin as the standard.

4. Statistical Analysis

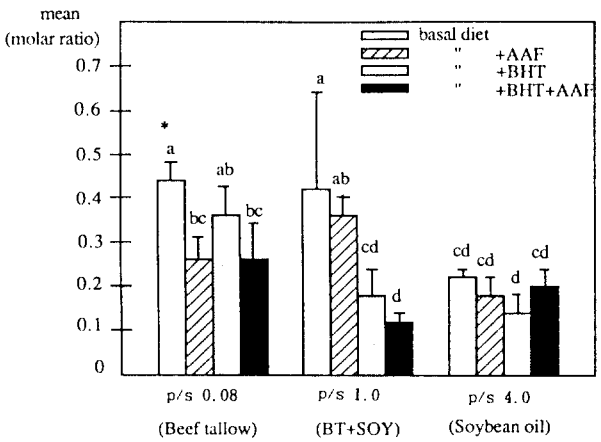
The results are presented as mean±SD. Comparison between individual diets was made by using Duncan's multiple-range test (SAS Institute, 1979).

III. RESULTS

1. Body and Liver Weights

No statistically significant differences in terminal body weights were observed among groups. However, higher liver wieghts were observed from the animals treated 2-AAF and BHT simultaneously than those fed basal diet in soybean oil diet.

2. Cholesterol/Phospholipid Molar Ratio in Hepatic Microsomes



* Means with the same letter are not significantly different ($p < 0.05$)

Fig. 2. Effect of dietary fats and BHT on the hepatic microsomal cholesterol/phospholipid ratio in AAF treated rats.

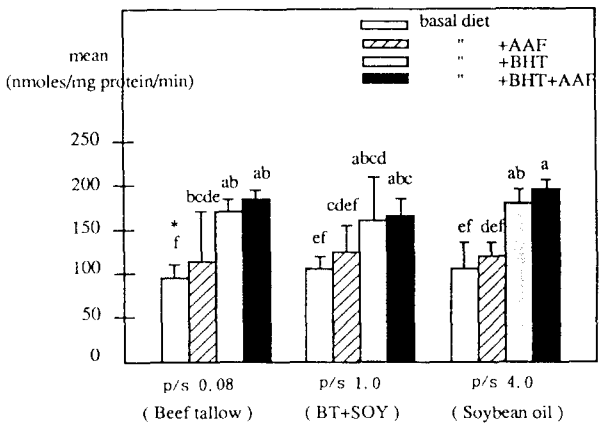
Fig. 2 depicts the cholesterol/phospholiid (c/p) molar ratio. In basal diet groups, soybean oil (SOY) group exhibited lower c/p molar ratio than beef tallow (BT) and beef tallow plus soybean oil (BT+SOY) group. There were no sinificant differences among subgroups in soybean oil (SOY) group. Injection of 2-AAF (50 mg/Kg body weight) significantly lowered c/p molar ratio in BT group, while BHT reduced the proportion of cholesterol in BT+SOY group.

3. Microsomal Thiobarbituric Acid Reactive

Table 2. Effect of dietary fatty acids on the hepatic microsomal, thiobarbituric acid reactive substances (TBARS) levels cytochrome P-450, cytochrome b_5 contents in 2-AAF treated rats

Group	TBARS (MDA nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)
Beef tallow (BT)	0.727±0.039 c	0.489±0.039 bcd	0.214±0.081 de
BT+AAF	1.193±0.061 a	0.561±0.032 a	0.307±0.031 abc
BT+BHT	0.710±0.097 cd	0.499±0.063 abc	0.303±0.059 abcd
BT+BHT+AAF	0.936±0.166 bc	0.550±0.018 ab	0.288±0.043 abcd
BT+SOY	0.731±0.056 c	0.436±0.022 cd	0.172±0.072 e
BS+AAF	1.035±0.311 ab	0.547±0.060 ab	0.274±0.028 bcd
BS+BHT	0.684±0.139 cd	0.372±0.047 cd	0.270±0.068 bcd
BS+BHT+AAF	0.922±0.060 bc	0.529±0.017 abc	0.226±0.034 cde
Soybean oil (SOY)	0.905±0.047 bc	0.305±0.055 e	0.162±0.047 e
SOY+AAF	1.137±0.267 ab	0.431±0.047 cd	0.262±0.086 bcd
SOY+BHT	0.471±0.040 d	0.475±0.040 abc	0.320±0.022 ab
SOY+BHT+AAF	0.690±0.089 cd	0.523±0.027 abc	0.377±0.059 a

Values are mean±SD.
Means with the same letter are not significantly different ($p < 0.05$).



* Means with the same letter are not significantly different ($P < 0.05$)

Fig. 3. Effects of dietary fats and BHT on the hepatic microsomal NADPH-cytochrome c reductase activity in AAF treated rats.

Substances(TBARS) Level

Microsomal TBARS level is also presented in Table 2. P/s ratio itself did not influence lipid peroxide level in this experiment. In SOY group, TBARS levels decreased significantly by BHT. Treatment of 2-AAF caused significant increase in the level of TBARS of all dietary group.

4. Microsomal Cytochrome P-450 Content

Microsomal cytochrome P-450 content is given in Table 2. In basal diet groups cytochrome P-450 content was lower in SOY group compared to those in groups BT and BT+SOY. Injection of 2-AAF increased the hepatic microsomal cytochrome P-450 content in all dietary groups. In SOY group, cytochrome P-450 content was significantly increased by feeding BHT or 2-AAF treatment.

5. Microsomal Cytochrome b_5 Content

Table 2 shows the microsomal b_5 content. Cytochrome b_5 content tends to have been lowered as p/s ratio increased, although the decrease was not significant. 2-AAF increased cytochrome b_5 content in all dietary groups. BHT also increased cytochrome b_5 content except in BT group.

6. Microsomal NADPH-Cytochrome C Reductase Activity

Fig. 3 presents the activity of microsomal NADPH-cytochrome c reductase. Dietary p/s ratio did not influence the enzyme activity. The enzyme activity increased only in BT group by 2-AAF treatment. BHT significantly increased enzyme activity in all dietary groups.

IV. DISCUSSION

In this paper, we have examined the effects of dietary fats and BHT on the hepatic microsomal

MFO system in 2-AAF treated rats. The microsomes in the liver is responsible for the oxidative metabolism (Remmer, 1970; Coon, 1978; Lu, 1976). The stability and permeability of membranes in the microsome depend on the phospholipids, and these characteristics of membranes could be influenced by dietary fatty acids (Zakim and Vessey, 1980). It is thus possible that dietary lipid alters the rate of oxidative metabolism by modifying the fatty acid composition of the microsome.

1. Cholesterol/Phospholipid Molar Ratio

An increase in the cholesterol/phospholipid (c/p) molar ratio may result in reduced membrane fluidity (Zakim and Vessey, 1980). Maloney *et al* (1986) showed that the age-related shifts in the c/p molar ratio correlated well with the changes in the fluidity of the microsomal lipid domain. In our experiment, 2-AAF and BHT+AAF lowered c/p molar ratio in BT group and BHT and BHT+AAF lowered it in BT+SOY group. This result agrees with those of Davison *et al* (1974) that infection of phenobarbitone (100 mg/kg per day) for 5 days caused decrease of the proportion of cholesterol in the microsomal membrane. In basal diet group c/p molar ratio was lower in SOY group than BT and BT+SOY group. It might be conjectured that the p/s ratio of dietary fat influenced the nature of microsomal membrane. It is also possible that when exogenous material is injected, fluidity of microsomal membrane tends to be increased to facilitate the entering of substances which are needed in metabolizing exogeneous material. Our previous experiment (Kim and Choi, 1992; Han *et al*., 1993; Kim, 1994) also showed that fatty acid composition in microsomal fraction was reflected by different dietary fats and microsomal linoleic acids were increased by 2-AAF treatment regardless of the diet. In this experiment, the c/p molar ratio was not reduced by 2-AAF and BHT in SOY group because longterm feeding of soybean oil have already lowered c/p ratio. Since dietary fat influences the biophysical properties of membrane, the modification of dietary fatty acids might make changes in cellular function to metabolize xenobiotics.

2. TBARS Levels

Lipid peroxidation in membranes occurs during many pathological as well as some physiological processes. TBARS are known to be primary products of the peroxidation process and it might cause more antioxidant deficient states or more carcinogenic condition (Wratten *et al*., 1992). In this experiment, microsomal TBARS level was not significantly influenced by dietary p/s ratio. Injection of 2-AAF raised the TBARS levels in all dietary groups. Oxygen free radicals are produced during the process of metabolic activation of xenobiotics by oxidase and peroxidase (Ames, 1984). Increased TBARS levels by 2-AAF were significantly lowered by BHT feeding in SOY group.

3. Microsomal Mixed Function Oxidase System

In agreement with previous researches (Astrom and Depierre, 1981; Astrom *et al*, 1983; Astrom *et al*, 1986), the results of our study showed that 2-AAF induced cytochrome P-450 synthesis. It has been noted by Cha and Heine (1982) that BHT increases cytochrome P-450 contents. In our experiment BHT increases cytochrome P-450 contents only in SOY group. Hammer and Wills (1979) reported that when a highly saturated coconut oil diet was fed to rats, the rate of oxidative demethylation of aminopyrine in the liver microsomes was less than when a corn oil diet was fed for 10 days. Norred and Wade (1972) also reported that microsomal drug metabolism and the content of cytochrome P-450 in hepatic microsomes were increased by feeding high levels of corn oil in the diet. Marshall and McLean (1971) obtained similar results as Norred and Wade (1972); they showed that the addition of herring oil or linoleic acid to the diet induced large increases in the concentration of cytochrome P-450 in response to phenobarbitone feeding. However, our results disagree with these studies. Hopkins and West (1976) observed that rats fed a diet containing tallow had greater concentrations of hepatic microsomal cytochrome P-450 than those fed a diet containing sunflower seed oil. Our study showed similar results as Hopkins and West (1976), since in our results cytochrome P-450 content was lower in SOY group than BT and BT+SOY group when rats fed basal diet. They explained these discrepancies in

such a way that greater amounts of TBARS in the diets used by other workers might be responsible for their results. It may be due to the differences in the composition and the duration of the diet as well. One of our previous studies (Yoon and Choi, 1990) showed that cytochrome P-450 contents and cytochrome c reductase activity were not increased by 2-AAF treatment in soybean oil group.

4. Butylated Hydroxytoluene

In this experiment, cytochrome c reductase activity was not influenced by the p/s ratio. BHT increased this enzyme activity in all dietary groups. A considerable number of experiments have shown that chemical carcinogenesis inhibited by the induction of microsomal enzymes (Wattenberg, 1978; Maikiura *et al.*, 1974). The decreased microsomal enzyme activity of rats fed the polyunsaturated fat may have enhanced their susceptibility to the action of chemical carcinogens. The results indicate that prolonged consumption of soybean oil diet appears to alter the lipid domain of microsomal membrane and thereby impair cytochrome P-450. The pattern of microsomal enzyme induction produced by BHT favours 2-AAF inactivation. Lu (1974) reported that in reconstitutional MFO system, cytochrome b_5 is not a determinant factor, but has an effect to control NADPH-dependant hydroxylation process. In this experiment, cytochrome b_5 content was observed to be lowered also as p/s ratio increased, although the changes were not significant. The changes of cytochrome b_5 contents in our experiment were similar to those of cytochrome P-450 contents.

In conclusion, long term intake of soybean oil diet changed the nature of microsomal membrane by lowering microsomal c/p molar ratio and induced less cytochrome P-450. Low level (0.3% of diet) of BHT increased cytochrome c reductase activity and lowered microsomal TBARS levels which were increased by 2-AAF. BHT might influence carcinogenic process by increasing xenobiotic metabolism and lowering lipid peroxidation. The significance of the change in microsomal membrane and the mechanism of how BHT affects hepatic MFO system activity are not fully understood yet. However, the potential anticarcinogenic effects of antioxidant supplementation and the overload of unsaturated fatty acids (soybean oil) on the composition and function of hepatic membrane should not be underestimated.

ACKNOWLEDGEMENT

This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1993.

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