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Effects of Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) on the Acetylation of 2-Aminofluorene and DNA-2-Aminofluorene Adducts in the Rat

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The effects of the synthetic phenolic antioxidants (butylated hydroxyanisole and butylated hydroxytoluene) on the *in vivo* acetylation of 2-aminofluorene and formation of DNA-2-aminofluorene adducts were investigated in male Sprague-Dawley rats. For *in vitro* examination, cytosols and intact cells, with or without butylated hydroxyanisole and butylated hydroxytoluene co-treatment, showed different percentages of 2-aminofluorene acetylation and DNA-2-aminofluorene adducts. For *in vivo* examination, pretreatment of male rats with butylated hydroxyanisole and butylated hydroxytoluene (10 mg/kg) 48 h prior to the administration of 2-aminofluorene (50 mg/kg) resulted in 34% and 18%, 29% and 20% decreases, respectively, in the urinary and fecal recovery of N-acetyl-2-aminofluorene, and 34% and 19% decreases, respectively, in the metabolic clearance of 2-aminofluorene to N-acetyl-2-aminofluorene. Following exposure of rats to the 2-aminofluorene, with or without pretreatment with butylated hydroxyanisole and butylated hydroxytoluene, DNA-2-aminofluorene adducts were observed in the target tissues of liver and bladder, and also in circulating leukocytes. The DNA-2-aminofluorene adducts in liver, bladder, and leukocytes were decreased by pretreatment with butylated hydroxyanisole and butylated hydroxytoluene. This is the first demonstration that synthetic phenolic antioxidants decrease the N-acetylation of carcinogens and formation of DNA-carcinogen adducts *in vivo*.

Key Words: **butylated hydroxyanisole (BHA); butylated hydroxytoluene (BHT); N-acetyltransferase (NAT) activity; arylamine toxicity; rats.**

Exposure to environmental and occupational carcinogens is recognized as a major factor in the initiation of human cancers. Benzidine, β-naphthylamine, and aniline derivatives have long been recognized as occupational arylamine carcinogens. Additional environmental arylamine carcinogens include 4-aminobiphenyl and 2-aminofluorene, found in combustion gases (Hoffman *et al*., 1969; Kuller *et al*., 1986). A major metabolic pathway for arylamines is *N*-acetylation, which is catalyzed by host cytosolic arylamine N-acetyltransferase (NAT), using acetyl coenzyme A as a co-factor (Weber and Hein, 1985). 2-Aminofluorene (AF) is *N*-acetylated to N-acetyl-aminofluorene (AAF), which can undergo further metabolism by cytochrome P450 enzymes, to reactive electrophiles capable of forming a variety of DNA and protein adducts. Deacetylation may play a role in modulating the toxicity of AF. Microsomal deacetylation of arylamines has been shown to generate mutagenic products in rabbit *in vitro* (Aune *et al*., 1985). Following exposure of mice to AF, DNA-AF adducts are found in several tissues including liver, bladder, and circulating leukocytes (Levy, 1993; Levy *et al*., 1994; Levy and Weber, 1992). NAT is found in many tissues of laboratory animals and humans (Chung *et al*., 1993; Hein *et al*., 1982; Juberg *et al*., 1991; Vatsis and Weber, 1993; Weber *et al*., 1976). Thus, the acetylation of carcinogenic arylamines is involved in determining organ- or tissue-specific susceptibility to cancer.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), the synthetic phenolic antioxidants, are effective in inhibiting carcinogen-induced tumor development in rodents (Wattenberg, 1972; Williams *et al*., 1986). BHT has also been reported to cause dose-dependent reduction of N-2-fluorenylacetamide-induced liver cancer and enhancement of bladder cancer in rats (Maeura *et al*., 1984). One of the actions of these phenolic antioxidants is inducing specific enzymes in the P450 complex. Ito *et al*., (1982, 1983) demonstrated that 2% and 0.5% dietary BHA in rats led to forestomach tumors. It has been demonstrated in a number of initiation-promotion type experiments that BHA and BHT can act as promoting agents (Ito *et al*., 1986). Other studies also found that BHT increased hepatocarcinogenesis in B6C3F1 mice (Inai *et al*., 1988). BHT has been considered a cancer chemopreventive agent, based on its ability to inhibit various phases of the carcinogenic process such as bioactivation and binding of carcinogenic chemicals to DNA (Slaga and Bracken, 1977; Wattenberg, 1980). Other investigators have demonstrated that BHT enhances the mutagenicity of, and DNA binding by, 3,3′-dichlorobenzidine (Ghosal and Iba, 1992). BHA and BHT also have important effects on detoxification pathways that affect GSH levels and induction of GSTs.

The effect of BHA and BHT *per se* on acetylation of

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carcinogens has not been previously reported. Therefore, the aim of the present study was to elucidate the possible effects of BHA and BHT on acetylation of carcinogen AF and the formation of DNA-AF adducts in the rat. The choice of tissues was based on the importance of liver in total acetylation activity in mammals. The bladder is a target tissue for arylamines in mammals, and is reported to differ in susceptibility to DNA damage (Levy and Weber, 1992). Lung is also a target tissue for arylamine carcinogens (e.g., methylene-bis(2-chloroaniline), 6-aminochrysene). DNA adducts in circulating leukocytes of mice have been used as indicators of exposure to AF (Levy, 1993). The results demonstrate that exposure to AF *in vivo* in combination with BHA and BHT resulted in decreased acetylation of AF and DNA-AF adducts *in vivo*.

MATERIALS AND METHODS

Chemicals and reagents. BHA, BHT, ethylenediaminetetraacetic acid (EDTA), leupeptin, 2-aminofluorene (AF), acetyl carnitine, N-acetyl-aminofluorene (AAF), carnitine acetyltransferase, dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), Tris, bovine serum albumin (BSA), and acetyl-Coenzyme A were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, dimethyl sulfoxide (DMSO), and potassium phosphates were from the Merck Co. All chemicals used were reagent grade.

Animals and treatment, in vitro assays. Male Sprague-Dawley rats weighing 180–200 g were obtained from stock maintained at the Animal Center of China Medical College. Rats were housed in cages and were maintained at 25°C on a 12-h light/dark cycle. Rats had free access to water and chow. All animals were at least 12 weeks-of-age at the time of killing. Blood was collected from the heart. Blood, liver, lung, and bladder were removed, and NAT activity was determined. Tissues from 6 rats were assayed individually for each activity determination.

Preparation of cytosols. Whole blood from the heart (50 μl) was hemolyzed immediately in 950 μl of lysis buffer [20 mM Tris–HCl, pH 7.5 (at 4°C), 1 mM DTT, 1 mM EDTA, 50 μM PMSF and 10 μM leupeptin]. The hemolysate was kept on ice (less than 30 min) until assayed for NAT activity. Blood leukocytes were isolated from Ficoll-Paque gradient centrifugation. Leukocytes (in 1 ml RPMI medium 1640 (GibcoBRL; Palsley, Scotland) with glutamine and 10% fetal calf serum) were incubated with AF at $\sim 1 \times 10^6$ cells/ml in individual wells of 24-well cell culture plates. They were co-treated with or without BHA and BHT for 18 h at 37°C in 95% air 5% CO_2. At the conclusion of incubation, the cells and media were removed and centrifuged. The cells were assayed for DNA-adduct formations, as described below. Liver, lung, and bladder tissues were each removed, trimmed, and placed in 5 volumes of the lysis buffer previously described. Tissues in lysis buffer were homogenized on ice with a Polytron homogenizer set at number 5 for 20 s (twice). The homogenates were centrifuged for 10 min at 100,000*g* and the supernatant kept on ice for NAT activity determination.

NAT activity determination. The determination of AcCoA-dependent N-acetylation of AF was as described by Chung *et al*., (1993). Incubation mixtures in the assay system brought it to a total volume of 100 μl: tissue cytosol, diluted as required, in 50 μl of lysis buffer (20 mM Tris/HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM acetylcarnitine) and 20 μl AF in DMSO at required concentrations for substrate. Then 10 μl BHA or BHT was added at various concentrations. Reactions were started by the addition of 20 μl of AcCoA. Control reactions had 20 μl water in place of AcCoA. The final concentration of AF was 0.1 mM, and that of AcCoA was 0.5 mM. The reaction mixtures were incubated at 37°C for 10 min and stopped with 100 μl of acetontrile for AF reactions. All reactions of experiments and controls were run in triplicate. The amounts of acetylated product and remaining nonacetylated substrate were determined by HPLC (Chung, 1998; Chung *et al*., 1993). An aliquot of the NAT incubation was injected onto a C18 reverse-phase column (Spherisorb 4.6 $\times$ 250 nm) of a Beckman HPLC (pump 168 and detector 126) and eluted at a flow rate of 1.2 ml/min. For AF and AAF, the solvent system was 20 mM KH_2PO_4, pH 4.5/CH_3CN (53:47) with detection at 280 nm. The retention time was 6.5 min for AAF and 9 min for AF. All compounds were quantitated by comparison of the integrated area of the elution peak with that of known amounts of standards. NAT activity is expressed as nmol acetylated per min per mg of cytosolic protein.

Protein determination. Protein concentration of the cytosols from the blood, liver, lung, and bladder were determined by the method of Bradford (1976) with bovine serum albumin as standard. All samples were assayed in triplicate.

In vivo examinations. Male Sprague-Dawley (SD) rats weighing 180–200 g were housed individual cages and were maintained at 25°C on 12-h light/dark cycle as described in *in vivo* studies (Chung *et al*., 1993). Gastric intubation was performed on each animal (Svensson and Knowlton, 1989), and BHA or BHT (0.1–100 mg/kg) was administered to each animal in isotonic saline (2 ml of saline administered) at a rate of approximately 0.5 ml/min. Control animals received isotonic saline containing no BHA or BHT. Twenty-four h later the rats were transferred to individual metabolism cages and AF was infused through the venous cannulae (maxillary veins) under anesthetic with halothone (60 mM AF in isotonic saline, 1% DMSO) at a rate of 0.5 ml/min over 60 min. An aliquot of the dosing solution (AF) was frozen and assayed for AF content. Serial blood samples (200 μL) were obtained through the cannula prior to and 5, 10, 20, 40, 80, 120, 160, 240, and 300 min after AF administration. Plasma (heparin for anti-coagulant) was separated by centrifugation (10 min at 600 *g*) and stored in polypropylene tubes at –20°C until analyzed. Urine and feces were collected in the dark through 52 h and immediately extracted twice with ethyl acetate/methanol (95:5), the solvent evaporated, and residue redissolved in methanol and assayed. AF and AAF were quantitated by HPLC as described in above.

Determination of the amounts of AF and AAF. The amounts of acetylated product and remaining nonacetylated AF were determined by HPLC (Chung, 1998, Chung *et al*., 1993) as described in above section.

Analysis of systemic clearance. The percentage urinary recovery of AF or AAF was calculated as the total amount of AF or AAF excreted in the urine within 48 h divided by the dose of AF treatment. The renal clearance (CLr) of AF and AAF were calculated as below equations (Svensson and Knowlton, 1989):

$$CL_r(AF) = \frac{\text{Total amount of AF excreted in urine}}{AUC(AF)}$$

$$CL_r(AAF) = \frac{\text{Total amount of AAF excreted in urine}}{AUC(AAF)}$$

The partial metabolic clearance of AF to AAF (CL_m) was calculated as the product of the percentage of the dose recovered in the urine as AAF and the systemic clearance of AF (Svensson and Knowlton, 1989).

Detection and measurement of DNA adducts. Control, BHA- or BHT-treated rats were sacrificed at a time of 3, 6, 12, and 24 h. Blood, liver, lung, and bladder tissues were isolated for DNA adduct determination. The DNA was prepared using G NOME DNA isolation kit protocol (BIO 101, La Jolla, CA). Then the genomic DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was hydrolyzed to 3′-monophosphate by spleen phosphodiesterase II and micrococcal nuclease (Levy and Weber, 1988; Levy *et al*., 1994). Five μg DNA (each) in 5 μl of TE buffer was mixed with 5 μl of 10$\times$ T4 polymerase buffer (0.33 M Tris acetate, 0.66 M potassium acetate, 0.10 M magnesium acetate, 5.0 mM DTT, pH 7.5) and 40 μl H_2O. Seventeen pmol of γ-[^{32}p]-dATP (3000 Ci/mmol) along with 22 units of T4 DNA polymerase were added to the above reaction buffer, and incubated at 30°C for

TABLE 1
Effects of BHA on N-Acetyltransferase Activity *In Vitro*

BHA treatment	AAF			
	Blood	Liver	Lung	Bladder
Control	0.30 ± 0.08	1.38 ± 0.24	0.74 ± 0.20	0.52 ± 0.10
0.08 μM	0.28 ± 0.08	1.34 ± 0.22	0.70 ± 0.26	0.54 ± 0.14
0.8 μM	0.20 ± 0.07[a]	1.18 ± 0.18	0.62 ± 0.14	0.40 ± 0.10[b]
8 μM	0.15 ± 0.06[a,b]	0.91 ± 0.14[a,b,c]	0.46 ± 0.10[a,b,c]	0.24 ± 0.08[a,b,c]
80 μM	0.08 ± 0.04[a,b,c]	0.26 ± 0.10[a,b,c,d]	0.18 ± 0.06[a,b,c,d]	0.12 ± 0.06[a,b,c]
160 μM	0.01 ± 0.02[a,b,c,d]	0.06 ± 0.02[a,b,c,d]	0.04 ± 0.02[a,b,c,d]	0.01 ± 0.02[a,b,c,d]
F value	32.90*	112.01*	38.18*	60.44*

Note. Values are mean ± SD of activity (nmol/min/mg protein); $n = 6$. Anova analysis used for comparisons.

* $p < 0.001$.

[a] Significantly different, at p level of 0.05, when compared to control.

[b] Significantly different, at p level of 0.05, compared to 0.08 μM

[c] Significantly different, at p level of 0.05, compared to 0.8 μM

[d] Significantly different, at p level of 0.05, compared to 8 μM

TABLE 2
Effects of BHT on N-Acetyltransferase Activity *In Vitro*

BHT treatment	AAF			
	Blood	Liver	Lung	Bladder
Control	0.30 ± 0.08	1.38 ± 0.24	0.74 ± 0.20	0.52 ± 0.10
0.08 μM	0.31 ± 0.10	1.36 ± 0.24	0.72 ± 0.24	0.50 ± 0.12
0.8 μM	0.22 ± 0.08	1.21 ± 0.18	0.64 ± 0.16	0.42 ± 0.10
8 μM	0.17 ± 0.06[a,b]	0.96 ± 0.15[a,b,c]	0.49 ± 0.12[a]	0.28 ± 0.08[a,b,c]
80 μM	0.10 ± 0.05[a,b,c]	0.37 ± 0.12[a,b,c,d]	0.22 ± 0.08[a,b,c,d]	0.16 ± 0.06[a,b,c]
160 μM	0.04 ± 0.02[a,b,c,d]	0.10 ± 0.04[a,b,c,d,e]	0.09 ± 0.04[a,b,c,d]	0.04 ± 0.02[a,b,c,d]
F value	20.71*	114.76*	25.31*	49.53*

Note. Values are mean ± SD of activity (nmol/min/mg protein); $n = 6$. Anova analysis used for comparison.

* $p < 0.001$.

[a] Significantly different, at p level of 0.05, compared to control.

[b] Significantly different, at p level of 0.05, compared to 0.08 μM.

[c] Significantly different, at $p$level of 0.05, compared to 0.8 μM.

[d] Significantly different, at p level of 0.05, compared to 8 μM.

[e] Significantly different, at p level of 0.05, compared to 80 μM.

60 min. The reaction was stopped by the addition of 10 μl of 0.5 M EDTA. The incorporated and unincorporated (free) isotopes were separated by Sephadex G-50 chromatography. Postlabeled adducted nucleotides were separated by Beckman HPLC (pump 168 and detector 126) using Ultrasphere C18 reversed phase ion-pairing column 4.6 × 25 cm eluted at a flow rate of 1.5 ml/min with 30 mM KH_2PO_4, pH 6.0, containing 10% CH_3CN, for 10 min followed by a linear gradient of 90% 30 mM KH_2PO_4, pH 6.0, 5 mM tetrabutylammonium phosphate and 50% CH_3CN at 65 min. UV absorbance followed at 254 nm. Samples (1 min = 1.5 ml) were collected and quantitated by scintillation spectrometry (Levy and Weber, 1988). Calculation of adduct formation was made by dividing the radioactivity at the adduct peak (after correction for recovery and efficiency of counting) by the specific activity of the ATP used in labeling. Adduct levels are reported as pmol adduct/mg of DNA analyzed (Levy and Weber, 1988).

Data Analysis. All results were summarized as mean ± SD. Means were compared by Anova analysis, or unpaired Student's t-test as appropriate. Some data were analyzed using the LAGRAN program, to obtain noncompartmental pharmacokinetics parameters (Rocci *et al*., 1983).

RESULTS

In Vitro

The possible effects of BHA and BHT on the NAT activity in rat blood, liver, lung, and bladder *in vitro* were examined by high pressure liquid chromatography assessing the percentage of acetylation of AF. Cytosols of blood, liver, lung, and bladder, with or without various concentrations of BHA or BHT co-treatment, showed different percentages of AF acetylation. A comparison of the relative cytosolic NAT activity, with or without various concentrations of BHA and BHT, are presented in Tables 1 and 2. The data indicate that there was decreased NAT activity associated with increased BHA and BHT in blood, liver, lung, and bladder cytosol, i.e., the higher the concentrations of BHA and BHT in the reaction mixture, the higher the inhibition of NAT activity. The 50% inhibition concentrations (IC_{50}) of BHA and BHT for blood, liver, lung, and bladder cytosol NAT activity were 8, 80, 8, and 8 μM, respectively.

The Michaelis-Menten parameters for NAT activity in blood, liver, lung, and bladder, with or without BHA or BHT pretreatment, are present in Table 3. The liver showed by far the greatest activity, while the blood showed the least. The Km values differed in the liver and other tissues. BHA or BHT treatment decreased apparent values of Vmax in 4 of the examined tissues (the exception was blood), but did not affect Km (Table 3).

TABLE 3
Michaelis-Menten Parameters for NAT Activity in Various Organs from Control, BHA and BHT Treated Rats

Organ	Treatment	Km (μM)	Vmax (nmol/min/mg protein)
Blood	Saline	420 ± 47	0.92 ± 0.14
	BHA	409 ± 40	0.84 ± 0.10
	BHT	412 ± 44	0.88 ± 0.10
Liver	Saline	188 ± 28	14.12 ± 1.88
	BHA	182 ± 30	9.6 ± 1.36[a]
	BHT	186 ± 32	9.8 ± 1.48[a]
Lung	Saline	271 ± 40	2.48 ± 0.43
	BHA	274 ± 44	1.67 ± 0.30[b]
	BHT	270 ± 40	1.84 ± 0.34[b]
Bladder	Saline	392 ± 44	1.29 ± 0.19
	BHA	398 ± 46	0.87 ± 0.12[c]
	BHT	393 ± 46	0.80 ± 0.12[c]

Note. Values are mean ± SD; $n = 6$. The kinetic constants were calculated from the modified HYPER Program of Cleland (1967).

[a] Significant difference between BHA or BHT and control ($p < 0.01$) by Student's t-test.

[b] Significant difference between BHA or BHT and control ($p < 0.05$) by Student's t-test

[c] Significant difference between BHA or BHT and control ($p < 0.05$) by Student's t-test

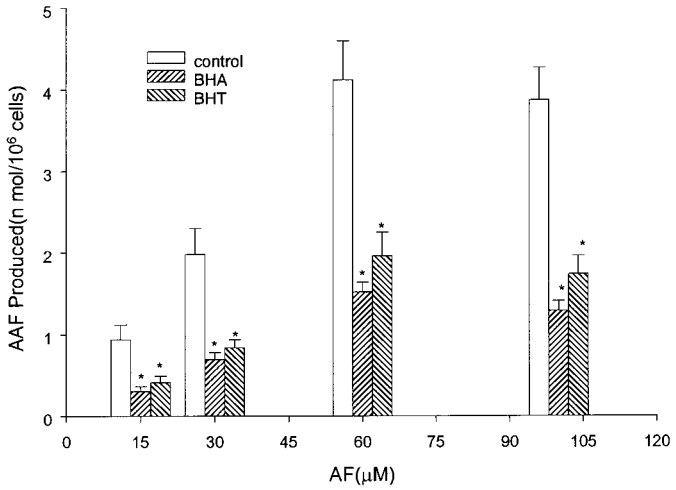

FIG. 1. Effect of BHA or BHT on production of AAF by rat leukocytes. Leukocytes were incubated as described for 18 h at the concentrations of AF co-treatment with and without 80 μM BHA or BHT. AAFs measured by HPLC assay. Each point represents the mean of triplicate assays of 3 incubations of cells. Bar, mean $\pm$ SD; $n = 6$; * $p < 0.001$.

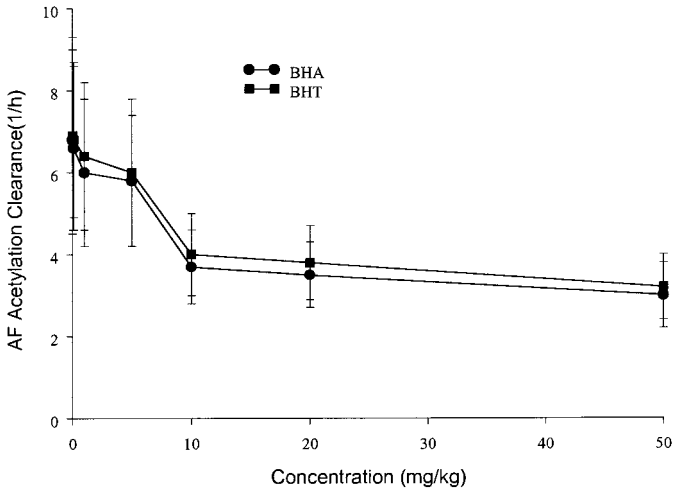

FIG. 2. Effect of BHA and BHT on the clearance of 2-aminofluorene via acetylation in rats. Animals were treated with 0.1–100 mg/kg BHA and BHT for 24 h. Data are expressed as mean $\pm$ SD; $n = 8$.

The NAT activity for the acetylation of AF (15, 30, 60, and 100 μM) measured from leukocytes (intact cells) was 0.94 $\pm$ 0.18, 1.98 $\pm$ 0.32, 4.12 $\pm$ 0.48, 3.87 $\pm$ 0.40 nmol/10^6 cells for acetylation of AF without co-treatment with BHA and BHT (Fig. 1). In the presence of 80 μM of BHA and BHT, the NAT activities were decreased about 64–69 and 53–58%, respectively (Fig 1).

Following the 18-h incubation of rat leukocytes with AF, co-treated with or without BHA or BHT, cells were recovered and DNA was prepared and hydrolyzed to nucleotides; adducted nucleotides were extracted into butanol and analyzed by HPLC (Levy *et al.*, 1994). The results indicate that leukocytes activate AF to a metabolite able to bind covalently with DNA (Table 4). In the presence of 80 μM BHA or BHT, the AF-DNA adduct formation decreased in both AF concentrations. Based on the data from *in vitro* studies, this indicates that 80 μM BHA or BHT had induced inhibition of NAT activity up to 50%. Thus, 80 μM BHA or BHT was selected for intact leukocyte studies.

In Vivo

Figure 2 illustrates the dose-related decrease in the disposition of AF acetylation by BHA and BHT treatment. Up to 1 mg/kg BHA and BHT did not significantly alter the acetylation rate of the AF. However, 10 mg/kg BHA and BHT significantly decreased the disposition of AF acetylation by 56%, and higher doses produced a similar effect. Therefore, 10 mg/kg BHA and BHT were selected for all experiments.

The mean plasma AF concentration (mean $\pm$ SD) versus the time profile after intravenous dosing of 50 mg/kg of AF in control rats and those pretreated with a single dose of 50 mg/kg BHA and BHT for 48 h is presented in Figure 3. The mean data obtained after the 240-min sample are not plotted because plasma AF concentration was below the limit of detection of HPLC by these time points. After the treatment of AF in control and BHA- or BHT-treated rats, it was discovered that the longer the experiments, the lower the amounts of AF in plasma concentration. The mean plasma AAF concentrations (mean $\pm$ SD) in control and BHA- or BHT-pretreated rats are given in Figure 4. The data indicate that rats treated with BHA or BHT had lower AAF concentration levels in plasma when compared to the control rats.

The pharmacokinetic parameters for AF in control, BHA-, and BHT-pretreated rats are presented in Table 5. There were no significant differences in the pharmacokinetic parameters for AF between the normal and pretreated (with BHA and

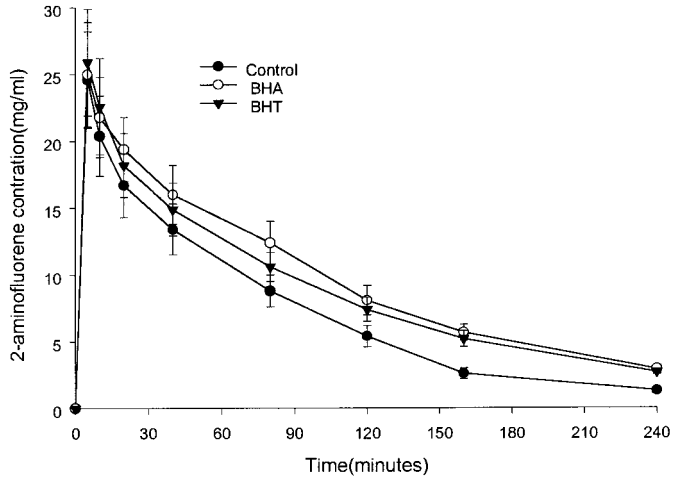

FIG. 3. Mean plasma 2-aminofluorene concentration versus time profile in BHA- and BHT-pretreated and control animals. The data were obtained as described in Materials and Methods. Values are mean $\pm$ SD; $n = 8$.

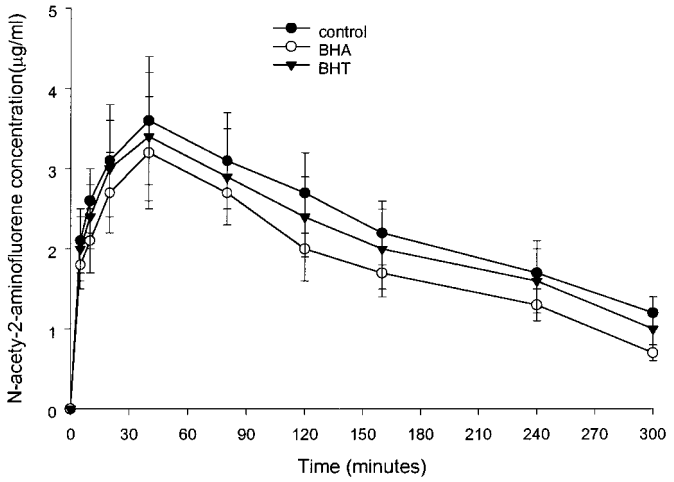

FIG. 4. Mean plasma N-acetyl-2-aminofluorene concentration versus time profile in BHA- and BHT-pretreated and control animals. The data were obtained as described in Materials and Methods. Values are mean ± SD; $n = 8$.

BHT) groups based on the values of CL_s (ml/min/kg), $t_{1/2}$ (min), and $V_{ss(}$liters/kg). The urinary recovery of AF and AAF in control and BHA- and BHT-pretreated groups are also shown in Table 5. The percentage urinary recovery of AAF was decreased in the BHA- and BHT-pretreated rats by 34% and 18% (from 12.4 ± 2.0 to 8.2 ± 1.1 and 10.2 ± 1.0; $p < 0.05$). In contrast, the urinary recovery of AF was not significantly changed by BHA and BHT pretreatment (Table 5). The fecal recovery of AF and AAF in control and BHA and BHT pretreated groups are also shown in Table 5. The percentage fecal recovery of AAF was decreased in the BHA and BHT-pretreated rats by 29% and 20% (from 17.4 ± 2.6 to 12.2 ± 1.4 and 14.0 ± 1.2; $p < 0.05$). In contrast, the fecal recovery of AF was not significantly changed by BHA and BHT pretreatment (Table 5). Data in Table 5 also showed that the renal clearance of AF and AAF did not significantly differ between control and

TABLE 4
Effects of BHA and BHT on DNA Adduct Levels in Rat Leukocytes

	DNA adduct formation (pmol adduct/mg DNA)	
Treatment	30 μM AF	60 μM AF
Control	1.38 ± 0.29	2.44 ± 0.48
BHA	0.34 ± 0.08[a]	0.87 ± 0.12[a]
BHT	0.51 ± 0.12[b]	1.29 ± 0.18[b]

Note. Values are mean ± SD of 6 separate preparations (isolation of leukocytes, incubation with 30 or 60 μM AF, co-treatment (or not) with 80 μM BHA or BHT, DNA preparation, post-labeling, and HPLC analysis).

[a] Significant difference between BHA and control. $p < 0.01$.

[b] Significant difference between BHT and control. $p < 0.01$.

TABLE 5
Effect of BHA and BHT Pretreatment on the Disposition of 2-Aminofluorene in Rats

Pharmacokinetic parameter	Control ($n = 6$)	BHA ($n = 6$)	BHT ($n = 6$)
CL_s (ml/min/kg)	114 ± 27	111 ± 18	114 ± 20
$t_{1/2}$ (min)	49.6 ± 5.2	44.9 ± 5.8	43.6 ± 5.9
V_{ss} (liters/kg)	4.86 ± 2.4	4.74 ± 1.8	4.82 ± 1.6
Urinary recovery (% of dose)			
2-Aminofluorene	32.7 ± 6.9	34.8 ± 6.0	35.9 ± 6.6
N-acetyl-2-aminofluorene	12.4 ± 2.0	8.2 ± 1.1[a]	10.2 ± 1.0[a]
Fecal recovery (% of dose)			
2-Aminofluorene	42.9 ± 7.4	45.6 ± 6.2	44.2 ± 6.0
N-acetyl-2-aminofluorene	17.4 ± 2.6	12.2 ± 1.4[b]	14.0 ± 1.2[b]
CL_r (AF) (ml/min/kg)	81.6 ± 25.2	84.2 ± 18.6	84.4 ± 17.9
CL_r (AAF) (ml/min/kg)	29.8 ± 8.4	28.4 ± 10.0	29.4 ± 12.2
CL_m (ml/min/kg)	15.6 ± 2.6	10.2 ± 2.0[c]	12.5 ± 1.8[c]

Note. BHA or BHT (50 mg/kg) was administered as a single dose 24 h prior to 2-aminofluorene (50 mg/kg) administation. Data are expressed as mean ± SD. CL_s, systemic clearance; $t_{1/2,}$ half-life; V_{ss}, steady-state volume of distribution; CL_r (AF), renal clearance of 2-Aminofluorene; (CL_r AAF), renal clearance of N-acetyl-2-aminofluorene; $CL_{m,}$ metabolic clearance of 2-aminofluorene to N-acetyl-2-aminofluorene (Stevenson and Knowlton, 1989).

[a,b,c] Differs significantly from 160 μM BHA, 160 μM BHT, or control; $p < 0.05$.

BHA and BHT pretreated rats. The metabolic clearance of AF to AAF was decreased by 34% and 19% (from 15.6 ± 2.6 to 10.2 ± 2.0 and 12.5 ± 1.8; $p < 0.05$) in the BHA and BHT pretreated rats compared to the normal rats.

The DNA-AF adducts in leukocytes, bladder, lung, and liver were followed for up to 24 h after the 60 mg AF/kg exposure. The DNA-AF adducts were determined for leukocytes, bladder, lung, and liver, with or without BHA and BHT, and are shown in Figures 5 and 6. The data indicate that there was decreased DNA-AF adduct in leukocytes, bladder, lung, and liver tissue of rats pretreated with BHA and BHT ($p < 0.05$, unpaired Student′s *t*-test). DNA-AF adduct levels in liver are higher than that in lung, which is also higher than that in bladder, and again higher than that in leukocytes, at examined time points. All examined tissues showed a linear increase in adduct formation with increased AF.

DISCUSSION

This study has documented the inhibition of N-acetylation of AF by BHA and BHT *in vitro* and *in vivo* in rat. Numerous studies have shown the acetyl CoA-dependent arylamine NAT enzyme has been reported to exist in many kinds of experimental animals, including humans, and the NAT has been shown to be involved in some chemical carcinogenesis (Minchin *et al.*, 1992; Grant *et al.*, 1992). Rapid and slow acetylation have been demonstrated to be predisposing factors for the sensitivity of individuals to toxicity during exposure to

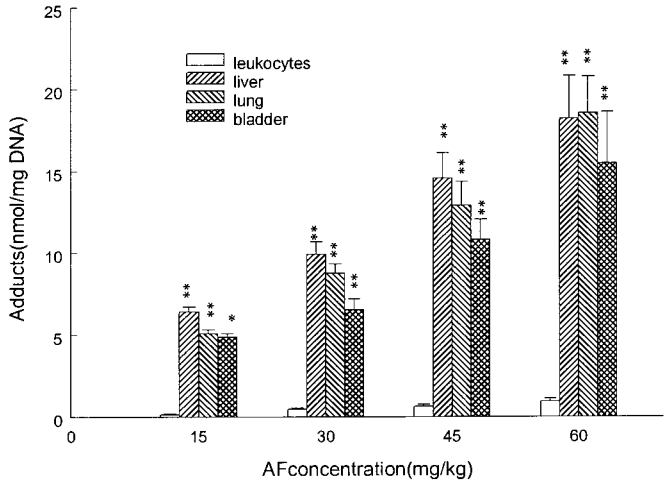

FIG. 5. DNA adduct levels in leukocytes, liver, lung, and bladder of SD rats 24 h after ip treatment in various doses of AF (15, 30, 45, and 60 mg/kg). Eight rats were used for each dose of AF treatment. Values are mean ± SD.

many arylamines (Weber, 1987; Weber and Hein, 1985). Therefore, the genetically mediated variation in NAT activities within target tissues for arylamine-induced neoplasms may indicate differential risk among the human population. The capacity to acetylate amino-containing compounds may determine an individual's predisposition to toxicity from various agents (Minchin *et al*., 1992). NAT activity is particularly abundant in the reticuloendothelial system (Grant *et al*., 1992; Minchin *et al*., 1992). Other investigators reported that the stimulation of the reticuloendothelial system by immunodulators reduces cytochrome P-450-dependent drug metabolism (Peterson and Renton, 1986). Elevated levels of NAT activity are associated with increased sensitivity to the mutagenic effects of many arylamine carcinogens. With the increase of NAT activity of *Salmonella typhimurium* strains comes the specific increase in their ability to detect the mutagenicity of aromatic, nitro, amino, and hydroxylamine components (Einisto *et al*., 1991). Attenuation of liver NAT activity is related to several disease processes such as breast and bladder cancer (Weber, 1987; Weber and Hein, 1985). The present studies demonstrated that BHA and BHT could decrease NAT activity in AF target and non-target tissues. BHA induced higher inhibition of NAT activity in the rat tissues than did BHT.

The reason for selecting 10 mg/kg BHA and BHT were based on two reasons: (1) dose-related studies (Fig. 1) already indicated that this concentration of BHA and BHT significantly decreased the disposition of AF acetylation by 56%; and (2) although the mean human dietary intake of BHA and BHT is 0.26 mg/kg/day (Kirkpatrick and Lauer, 1986), even 0.26 to 5 mg/kg BHA and BHT did not show any significant difference between control and BHA- and BHT-treated rats. The procedure of selecting pretreatment with BHA and BHT for 24 h and then adding AF was based on the report from Benson *et al*. (1984) demonstrating that one day of dietary BHA could significantly increase NAD(P)H: quinone reductase and glutathione transferase activities in the liver, kidney, and proximal small intestine, and NAD(P)H:quinone reductase-specific activity in the forestomach and lung. In the proximal small intestine, glutathione transferase-specific activities toward 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene rose to 2.6 and 8 times those of control, respectively, and NAD(P)H:quinone reductase-specific activity doubled within 1 day on the BHA diet. Thus, one day of administration may suffice to induce large changes in phase II enzyme activity, due to enzyme-induction effects. The data in the urinary and fecal recovery of AAF and the partial clearance of AF to AAF indicate that pretreatment with BHA and BHT results in an inhibition of arylamine carcinogen acetylation *in vivo*. The data did show that any change in the total clearance of AF, in the presence of a decrease in the partial metabolic clearance of AAF, is most likely caused by two different interactions by BHA and BHT. First, other reports had already shown that the elimination pathways of procainamide include renal excretion of unchanged drug, acetylation, and oxidative metabolism (Svensson and Knowlton, 1989). Second, the present data indicate a decrease in acetylation of AF and the total clearance of AF, which might suggest that BHA and BHT inhibit the oxidative metabolism of AF. This point requires further investigation.

The present results have shown that BHA and BHT inhibit arylamine carcinogen (AF) acetylation, but other reports have shown that other compounds increased acetylation of the drug. For example: (1) stimulation of the reticuloendothelial system by Freund's adjuvant led to an increased rate of N-acetylation *in vivo* in both the rat (Zidek *et al*., 1981) and the rabbit (DuSouich and Courteau, 1981); (2) pretreatement of tilorone resulted in an increased rate of N-acetylation of procainamide *in vivo* in the rat (Svensson and Knowlton, 1989); (3) chronic administration of hydrocortisone in the rabbit was shown to enhance the acetylation of sulfamethazine (Reeves *et al*., 1988); and (4) aspirin treatment resulted in a decrease in AF acetylation in rats (Chung, 1998). The present data also show that BHA and BHT decrease the renal clearance of AF or AAF, but other results from a Svensson and Knowlton (1989) study demonstrated that tilorone pretreatment had no effect on the renal clearance of procainamide or N-acetylprocainamide.

The present study also indicates that BHA and BHT pretreatment results in decreasing the formation of DNA-AF adducts during the acetylation of AF in rat leukocyte, liver, lung, and bladder tissues *in vivo*. The decrease in DNA adduct formation may be due to the decrease in acetylation of AF. However, other reports have demonstrated that BHA decreases AF-DNA adduct formation in the intact polymorphonuclear leukocytes due to the inhibition of the respiratory burst or interference with DNA binding by peroxidized AF metabolites (Tsuruta *et al*., 1985). This study also shows that pretreatment of BHA and BHT in the rat leads to a decrease of AF acetylation. The mechanisms of BHA and BHT effects on DNA-AF

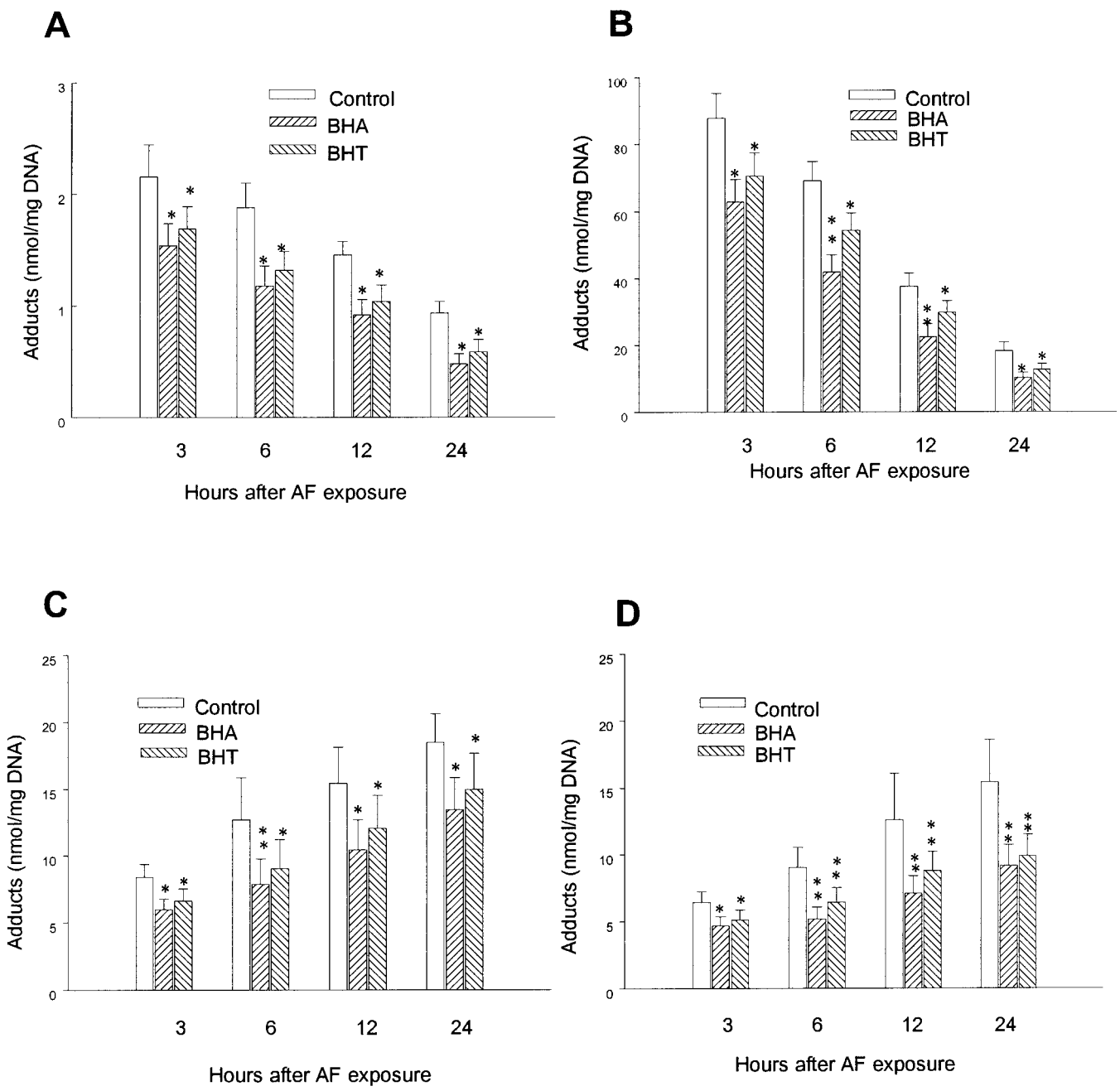

FIG. 6. DNA adduct levels in leukocytes, liver, lung and bladder of SD rats 3 to 24 h after ip dose of AF (60 mg/kg). Eight rats were used for each time point. Panel A represents leukocytes; panel B, liver; panel C, lung; and panel D, bladder. Values are mean ± SD.

adduct formation in the rat is not known at this time, and further investigation is needed. The DNA-AF adduct levels show variation among rat tissues, with liver >lung > bladder > leukocytes in with-or-without BHA and BHT pretreatment of the animals. This may be due to the original NAT activity difference in those examined tissues. The AF-NAT activity shows variation among the tissues with liver > lung > bladder > blood in normal SD rats (data not shown). Although the present study demonstrates that DNA-AF adducts in leukocytes, liver, and bladder are slightly lower than results in mice from other previous reports (Levy, 1993; Levy and Weber, 1989, 1992,), the DNA-AF adduct formation with carcinogen exposure-time trend is similar. The present data also show that DNA-AF adducts occurred in the lung. In other words, DNA adduct formation in the lung was similar to that found in liver, leukocytes, and bladder, but the liver and bladder contained a higher adduct profile. All four tissues showed a linear increase in adduct formation with increased

dosage of AF (Fig. 5). DNA-AF adducts were observed within 3 h after a larger dose of AF. Following the adduct levels for 24 h indicated a fairly constant relationship between leukocyte and liver adducts and between lung and bladder adducts. The decline in leukocyte adducts was parallel to the decline in liver adduct levels. The rise in lung adducts was parallel to the rise in bladder adduct levels, but the DNA adducts in bladder and lung showed a linear increase over time. It is possible that the increase in bladder adducts is caused by the movement of AF metabolites from liver or other tissues to the bladder, followed by acid-catalyzed hydrolysis and reactivation to an ultimate carcinogen (Lang and Kadlubar, 1991). There are no previous reports about the DNA-AF adduct in lung tissues. Carcinogen DNA adduct formation is an important step in chemical carcinogenesis. In this study, BHA and BHT demonstrate that both phenolic antioxidants could decrease the DNA-AF adduct levels *in vivo*. This finding is in agreement with that of Richer *et al.* (1989) who showed inhibition of binding of 2-acetylaminofluorene to DNA by BHA and BHT *in vitro*. Similar results were also found by Chipman and Davies (1988) in human and rat hepatocytes. Therefore, it may be the reason why antioxidants can prevent cancer. Thus, the exact mechanism of BHA and BHT inhibition of DNA-AF adduct formation needs further investigation.

In conclusion, the results indicate that the antioxidants BHA and BHT decrease the rate of arylamine carcinogen acetylation and DNA-AF adduct formation in rats *in vivo*. The present results may also indicate that predisposition to carcinogen toxicity from agents might be influenced by antioxidants. Future studies will focus on the mechanism of this decrease of acetylation and its significance in the toxicity of agents that undergo biotransformation via acetylation.

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