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Effect of L-Ascorbic Acid and DL-α-Tocopherol on the Activation and Binding of 2-Acetylaminofluorene to Rat Liver Nuclear Macromolecules in Vivo

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Male Sprangue-Dawley rats which had been administered either ascorbic acid or DL-α-tocopherol for 4 weeks were injected intraperitoneally with a single dose of(9-^{14}C)-2-acetylaminofluorene(AAF) 3.5hr before sacrifice. The activation and binding of a hepatocarcinogen, AAF to the nucleic acids and proteins rat liver nuclei were examined. After the precipitated DNA was enzymatically hydrolyzed and the adduct fraction was purified by Sephadex LH-20 chromatography, the individuals adduts were separated by HPLC. the liver microsomal and nuclear monooxygenase activities in AAF administered rats were increased, whereas those in ascobric acid and DL-α-tocopherol fed rats were decreased. It is suggested that microsomal and nuclear monooxygenase were essential for activated AAF to bind nucleic acid under the intracellular conditions. The inhibitory ability of ascobic acid and DL-α-tocopherol on the binding of AAF to liver nuclear DNA was most probably due to the marked inhibition of the formation of the proximate carcinogen. The protective effect of ascorbic acid and DL-α-tocopherol against the hepatocarcinogenic action of AAF may be mediated by decreased monooxygenase activities and by alteration in the binding of the esterified AAF to liver nucleic acids.

Introduction

Most chemical carcinogens require metabolic activation by the microsomal electron transport system before they can bind covalently to target molecules. The covalent interaction of chemical carcinogens with DNA is considered to be a critical step in carcinogenesis[1~4], and it seems that microsome-activated carcinogens reach the nucleus to react with DNA.

It is reported that nuclear monooxygenase in rat liver cells is inducible[5]. The monooxygenase system in the nuclear envelope may increase the reaction between the activated metabolites of carcinogens and nuclear DNA.

Ascorbic acid, DL-α-tocopherol and selenium as well as butylated hydroxyanisole and butylated hydroxytoluene are antioxidants that have been shown to exert

a protective function against liver damage, aging, carcinogen-induced chromosomal breakage, mutagenesis and chemical carcinogenesis in experimental animals[6~11]. Some vitamins may act like synthetic antioxidants to alter either the metabolism of carcinogenic chemicals or their interaction with important tissue macromolecules[12].

Lake et al[6]. have found that L-ascorbic acid can inhibit the covalent binding of reactive acetaminophen metabolites to proteins in vitro.

We have investigated the role of the vitamin antioxidants, ascorbic acid and DL-α-tocopherol on the microsomal and nuclear monooxygenase system in the activation of AAF. This study has further investigated the effect of dietary ascorbic acid and DL-α-tocopherol on the covalent binding of AAF metabolites to rat liver nuclear macromolecules in vivo.

Materials and Methods

Animals : Young adult Male Sprague-Dawley strain rats, weighing 150 to 200 g each were used throughout this study and were given free access to food pellets and water. The rats, in groups of 5 or 6 animals, were given oral administration by stomach tube of either vitamin E(12 I.U/100g body weight in corn oil/day) ascorbic acid (10mg/100g body weight in saline/day) for 4 weeks. The rats were each given i.p. injections of AAF containing 2.5 μCi(9-^{14}C) AAF in 25 μl ethanol. Rats were killed 3.5hr after AAF administration, and DNA was isolated from their livers.

Chemicals: (9-^{14}C) AAF(Sp. activity 55.3mCi/mmol) was obtained from Amersham international LTd, England). L-Ascorbic acid and DL-α-tocopherol obtained from Merk CO. & Inc. West Germany.

N-Acetoxy-AAF was prepared by the acetylation of N-OH-AAF as described[13]

Reaction of N-acetoxy-AAF with calf thymus DNA and isolation of DNA adducts were as described by Beland et al[25].

In vivo carcinogen Binding: (9-^{14}C) AAF(55.3mCi/mmol) was administered at a dose of 0.428mmol/animal. Animals were sacrificed 3.5hr after injection. Isolation of nuclei, nuclear DNA, RNA and protein was according to the procedure of stout et al[21]. In brief, their liver homogenate prepared in 4 volumes of 0.15M KCl were resuspended in 10 volumes of 0.25M sucrose containing 10mM Tris and 1 mM $MgCl_2$ pH 7.8(STM). The suspension was filtered through cheesecloth, and the filtrate was centrifuged at 100 $\times$g for 10min. Pellets were resuspended in 10ml of 2M sucrose:1mM $MgCl_2$:10mM Tris, pH 7.8, and centrifuged at 100,000 $\times$g for 30min. in a Beckman SW 27 rotor. Nuclear pellets after being washed by resuspension in STM and centrifugation at 500 $\times$g for 10min. Were dissolved in 2ml of 5M urea containing 1mM EDTA:10mM Tris, pH 7.8, and the RNA was digested with RNase A(50 μg/ml) at 37° for 15min. Free carcinogen was extracted 3 times with 2 volumes of ethyl acetate. DNA and protein were precipitated from the aqueous phase by adding 4 volumes of ethanol and storing at -20° for 3hr or longer.

Differential extraction of DNA and protein was carried out by perchloric acid hydrolysis. Aliquots of DNA, RNA and protein were taken for radioactivity measurement and for quantitation[14][15].

DNA hydrolysis and adduct chromatography. DNA, dissolved in a convenient volume(ca 1mg/ml) of 5mM Bis-Tris, 0.1mM EDTA, pH 7.1, was made 10mM in magnesium chloride, heated for 3min in boiling water and rapidly cooled with ice. Deoxyribonuclease I(bovine pancrease, Sigma DN-Cl, 0.1mg/mg DNA) and endonuclease[16] (N. crassa, Sigma E4253, 6 units/mg DNA) were added and the solution was incubated overnight at 37°. The pH was than adjusted to 8.0 by addition of 1M Tris base (Sigma) and then phosphodiesterase I (Crotalus atrox, Sigma p6761, 0.04 units/mg DNA) and alkaline phosphatase (E. coli, Type IIIS, Sigma p4377, 1 unit/mg) were added. The incubation was continued overnight, after which the pH was readjusted to 7.0 with 1 N hydrochloric acid and the solution was stored at -20°.

In some cases following enzyme hydrolysis of DNA as described by Beland at al[25]. unlabeled deoxynucleoside adducts obtained from the reaction of N-oAc-AAF with calf thymus DNA were added to serve as UV markers for subsequent column chromatography.

For adduct separation, Sephadex and high performance liquid chromatography procedures were as described by Beland et al[25]. in brief after the aqueous solutions were passed through a Sephadex LH-20(1.6×20cm) column and eluted with water to remove deoxynucleosides, protein salts, the carcinogen-bound deoxynucleosides were eluted with methanol. The methanol fraction was concentrated and was then injected into a Waters Model. M-6,000A high performance liquid chromatograph, equipped with a U6K injector, a 440 UV detector, a 660 solvent programmer and a 10-μm μBondapak C_{18} column(30cm×3.9mm). The adducts were separated by isocratically running 56% methanol for ca. 20 min followed by a linear program to 100% methanol in 2 min at a flow-rate of 1 ml/min.

Analytical procedures. The cytochrome b_5 and P-450 contents of nuclei and microsomes were determined as described by Omura and Sato[17]. NADH - and NADPH-cytochrome c reductase were assayed as described by Omura and Takesue[18]. Lipid peroxidation was quantitated in terms of hepatic MDA content according to the procedure of Slater[19]. Protein was determined by the method of Lowry et al[14]. Using bovine serum albumin as a standard.

Results

Comparison of Enzyme Activities of Nuclei. Table 1 shows the enzyme activities of microsomes and nuclei of rat liver. When either ascorbic acid or DL-α-tocopherol was administered, the cytochromes P-450 and b_5 contents were decreased in comparison with the control group. In group treated with AAF and ascorbic acid or DL-α-tocopherol the cytochrome P-450 and b_5 levels were also much less than that observed after the treatment of AFF alone. With AAF treated rats, NADPH-cytochrome c-reductase and NADH-cytochrome c reductase activities were higher than those of the control rats. However, these enzyme activities were less from AAF and ascorbic acid or DL-α-tocopherol treated rats than those from rats AAF treated animals. The enzyme activity in the nuclear fraction was approximately one-half or one-third of that found in the microsomal fraction. Although these enzymes are known to be localized in the microsomes, they also

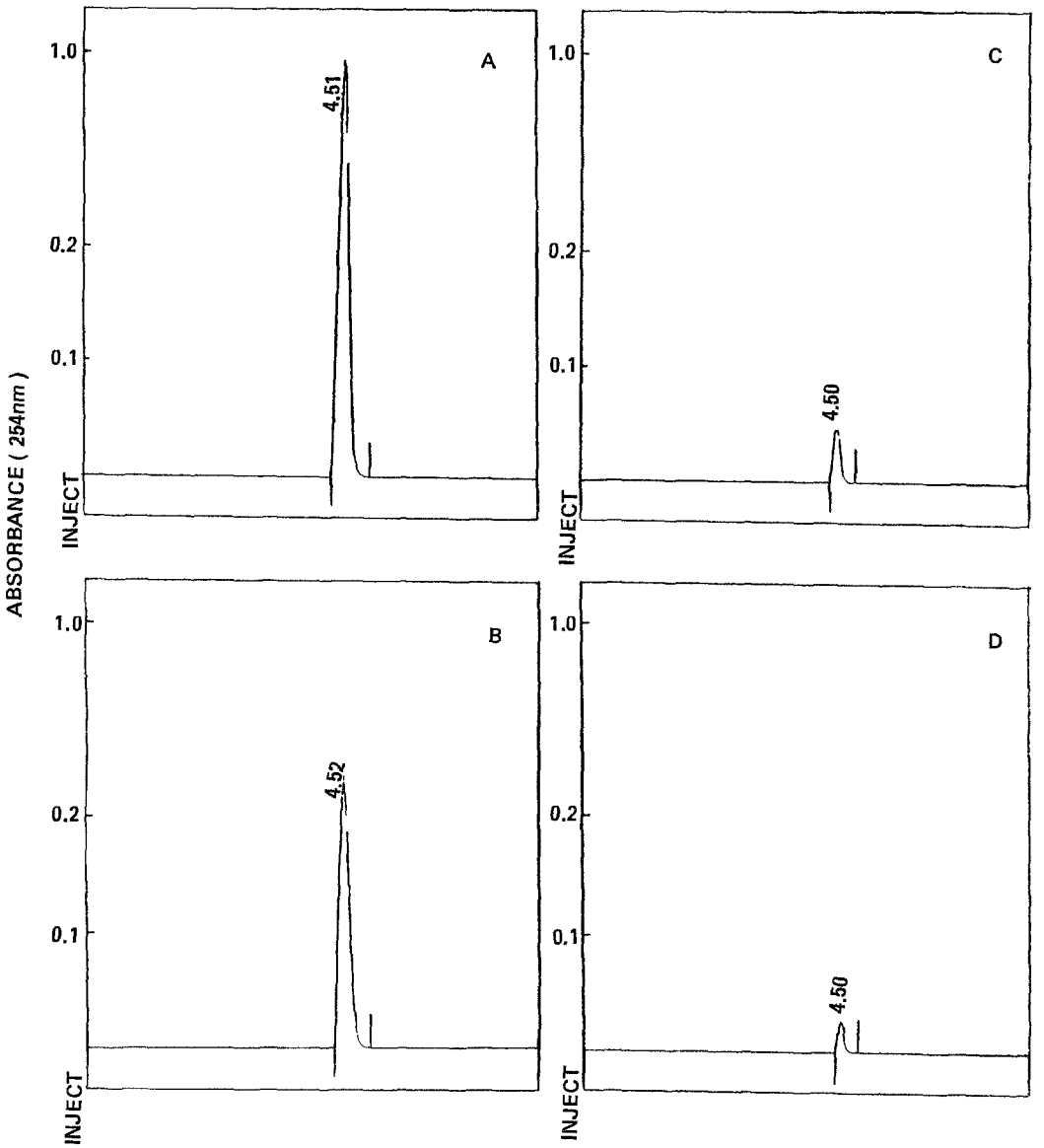

Fig. 1. HPLC profile of enzymatically hydrolyzed AAF bound rat liver microsomal DNA. Conditions : 10 μm μBondapak C_{18}, 30cm × 3.9mm; 100% methanol; Flow rate 1ml/min. A; N-OH AAF-calf thymus DNA, B; AAF, C; 12mg Vitamin E + AAF, D; 10mg Vitamin C + AAF.

showed significant activities in the nuclear fraction. The formation of lipid peroxides in ascorbic acid or DL-α-tocopherol fed rats was decreased in than that in the control. Also AAF with ascorbic acid or DL-α-tocopherol treated rats were decreased than that of the AAF treated rats.

Binding of AAF to nuclear DNA, RNA and protein. Maximum AAF binding in vivo has been reported to occur 2 to 4 hr after injection[20]. Therefore, 3.5hr after injection, nuclear DNA, RNA, and protein were isolated. Table 2 shows the effect of ascorbic acid DL-α-tocopherol pretreatment on the binding of AAF to liver

Table 1. Enzyme activities nuclei in rat liver

	NADPH-cytochrome C reductase nmoles/min/mg protein	Cytochrome P-450 nmoles/mg protein	NADH-cytochrome C reductase nmoles/min/mg protin	Cytochrome b_5 nmoles/mg protein	Lipid peroxides nmoles/ml/30min
Control	0 114 ± 0.05	1.682 ± 0.243	0.613 ± 0 010	0.033 ± 0.001	6.763 ± 0.409
Ascorbic acid	0.083 ± 0.003[a]	1.458 ± 0.160[b]	0.471 ± 0.003[a]	0.028 ± 0.001[a]	4.631 ± 0.008[a]
DL-α-tocopherol	0.096 ± 0.005[b]	2.079 ± 0.210	0.504 ± 0.003[a]	0.020 ± 0.001[a]	2.809 ± 0.077[a]
AAF	0.127 ± 0.005[b]	2.079 ± 0.210[b]	0.709 ± 0.006[a]	0.045 ± 0.003[a]	7.897 ± 0.036[a]
Ascorbic acid + AAF	0.105 ± 0.002[b]	1.638 ± 0.193	0.573 ± 0.044[c]	0.021 ± 0.003[c]	4.007 ± 0.400[c]
DL-α-tocopherol + AFF	0.097 ± 0.003[b]	1.605 ± 0.185	0.589 ± 0.010[c]	0.025 ± 0.003[c]	3.173 ± 0.032[c]

Each value representads mean ± S.D. of 6 experiments

[a] Significantly different from control value $P < 0.01$

[b] Significantly different from control value $P < 0.05$

[c] Significantly different from AAF value $P < 0.01$

nuclear macromolecules. Binding of AAF to DNA in ascorbic acid-treated group was decreased. The decrease in the binding was larger in DL-α-tocopherol treated group than in ascorbic acid-treated group. The amount of AAF bound to liver nuclear RNA and protein of ascorbic acid or DL-α-tocopherol fed rats was much less than that of the control group. These results confirm that ascorbic acid or DL-α-tocopherol pretreatment inhibit the binding of AAF to nuclear macromolecules(Fig. 1).

Discussion

We have investigated in this study the role of ascorbic acid and DL-α-tocopherol in the detoxification of the reactive metabolites that are generated from AAF in the rat in vivo by inhibition of nuclear DNA binding with AAF. The rationate for studying this aspect of the overall problem was based on 2 considerations. First dietary ascorbic acid[6] DL-α-tocopherol[11] has been shown to delay markedly the appearance of AAF-induced tumors in rat liver. Second, AAF has been extensively studied and is known to from covalent adducts at both the C-8 and N-2 positions of guanine in rat liver DNA in vivo[24)25)].

The experimental approach involved a comparison between the effects of AAF in rats fed either a ascorbic acid or DL-α-tocopherol for 4 weeks. Consequently, it was necessary to know the functional ascorbic acid and DL-α-tocopherol status of the animals prior to their being exposed to the carcinogen. This was achieved by measuring liver microsomes and nuclei monooxygenase activity. As shown in Table 1, liver microsomes and nuclei enzyme activities were decreased to observed after the treatment of AAF alone.

Bartsch and Hecker[26] and Floyd et al[27~29]. have shown that in the presence of either H_2O_2 or lipid hydroperoxides the proximate carcinogenic metabolite of AAF, i.e., N-hydroxy-2-acetylaminofluorene undergoes a 1-electron oxidation in vitro to yield nitroxide free radicals. The reaction is catalyzed by heme or heme-containing peroxidases and results in the formation of N-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene via the dismutation of 2-nitroxide free radicals.

Table 2. Effect of ascorbic acid or DL-α-tocopherol pretreatment on covalent binding of(9-^{14}C)-acetylaminofluorene to rat liver nuclear DNA, RNA and protein

Treatment	Binding of AAF to Liver Nuclear Macromolecule		
	p mol/mg DNA	p mol/mg RNA	p mol/mg Protein
None(control)	3.716 ± 0.070	3.285 ± 0.131	0.147 ± 0.021
Ascrbic acid	2.896 ± 0.467	2.363 ± 0.260[a]	0.107 ± 0.021[a]
DL-α-tocopherol	2.606 ± 0.307[a]	2.700 ± 0.387[b]	0.105 ± 0.029[a]

Animals were treated with ascorbic acid or DL-α-tocopherol as described in methods and materials. AAF-9-^{14}C was injected 3.5hr before sacrificed. All other details are described in materials and methode.

Each vale represents mean ± S.D. of 6 experiments

[a] Significantly different from control value $P < 0.001$

[b] Significantly different from control value $P < 0.01$

We measured the hepatic microsomes and nuclei malondialdehyde(MDA) content of rats in control groups and in dietary groups following exposure to AAF. MDA is a product of lipid peroxidation which reportedly possesses carcinogenic activity[30]. Moreover, Shamberger[31] found an increase in the MDA content of mouse skin following treatment with the carcinogen DMBA. The data in Table 1 show that the hepatic microsomal and nuclear MDA content were decreased in dietary groups. These results suggest that vitamin antioxidant is associated with decreased level of lipid peroxides which might potentially serve as substrates for the free radical activation of AAF.

The effect of dietary ascorbic acid and DL-α-tocopherol on the total binding of AAF to liver DNA in vivo is shown in Table 2. The data show the vitamin antioxidants inhibited the binding of the carcinogen to DNA. These results indicate that the protective effect of ascorbic acid or DL-α-tocopherol is mediated by an alteration in the binding of AAF to target tissue DNA. In this respect, the mechanisms underlying the anticarcinogenic action of vitamin antioxidant similar to those of the synthetic antioxidant butylated hydroxytoluene, which has been shown to decrese the binding of the carcinogen[8,32] to rat liver DNA.

In summary, the observations by others that ascorbic acid and DL-α-tocopherol inhibit tumor induction and the mutagenic effect of carcinogens such as AAF suggest that vitamin antioxidants may play a role in protecting DNA from the damage produced by the administration of AAF.

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