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Diallyl sulfide down-regulates polycyclic aromatic hydrocarbon-induced cytochrome P450 1A1 in mouse liver and lung

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Abbreviations: CYP, cytochrome P450; EROD, 7-ethoxyresorufin-*O*-deethylase

Abstract

The expression of cytochrome P450 genes directly within target cells is an important determinant of human susceptibility to cancers and other chemically initiated diseases. One pivotal gene, *CYP1A1* codes for an inducible cytochrome P450 isozyme 1A1 responsible for the bioactivation of numerous carcinogenic polycyclic hydrocarbons. In the present study, the effects of 3-methylcholanthrene, a polycyclic aromatic hydrocarbon, on the activity and expression of CYP1A1 and the protective effects of diallyl sulfide on 3-methylcholanthrene-induced changes in mice liver and lung were investigated. After a four daily 3-methylcholanthrene-treatment (25 mg/kg, i.p.), liver and lung microsomal 7-ethoxyresorufin-*O*-deethylase (EROD) activity, associated with CYP1A1, was increased. A corresponding increase in the level of CYP1A1 mRNA was observed in mouse liver and lung after 3-methylcholanthrene-treatment by Northern blot analysis. Diallyl sulfide reduced 3-methylcholanthrene-induced CYP1A1 mRNA expression and its associated EROD activity in mouse liver and lung. The modulation of CYP1A1 mRNA by 3-methylcholanthrene and diallyl sulfide was mainly due to transcriptional regulation.

Keywords: diallyl sulfide, cytochrome P450, carcinogen, 3-methylcholanthrene, mouse, liver, lung

Introduction

The cytochrome P450 (CYP) superfamily of isozymes is involved in the metabolism of a diverse range of compounds (Nebert *et al*., 1991). The CYP1A subclass of this family consists of two members, CYP1A1 and CYP1A2, which are responsible for the metabolism of many xenobiotic compounds, including drugs, carcinogens, mutagens, dietary components, and environmental pollutants (Black and Coon, 1987). Carcinogenic compounds, such as benzo(*a*)pyrene and benzanthracene are metabolized preferentially by CYP1A1, whereas aflatoxins and arylamines are metabolized by CYP1A2 (Aoyama *et al*., 1990). These enzymes are implicated in both carcinogen detoxification and activation (Butler *et al*., 1989a,b; Santhanam and Lotlikar, 1989; Aoyama *et al*., 1990). The polycyclic aromatic hydrocarbons have been shown to elicit prolonged biochemical responses in several rodent species, particularly induction of mixed function oxygenases in hepatic tissue. Lung is also of interest since polycyclic aromatic hydrocarbons have been demonstrated to have tumor promoting effect, increasing the numbers of lung tumors in Swiss mice initiated with *N*-nitrosodimethylamine.

Diallyl sulfide, a compound derived from garlic, has been shown to inhibit chemically induced carcinogenic and cytotoxic responses in rodents. Inhibition of the CYP enzymes responsible for the metabolic activation of the carcinogenic and toxic chemicals is believed to be a major mechanism of the observed chemopreventive effect of diallyl sulfide. diallyl sulfide has been shown to reduce the incidence of a variety of carcinogen-induced tumors, including those induced by polycyclic aromatic hydrocarbons. Investigations of these anticarcinogenic actions have demonstrated that diallyl sulfide inhibits the CYP-dependent activation of the polycyclic aromatic hydrocarbon benzo(*a*)pyrene. The present study was therefore designed to investigate the effects of 3-methyl-cholanthrene on the activity and expression of CYP1A1 and the protective effects of diallyl sulfide on 3-methylcholanthrene-induced changes. Results obtained from this study will be useful to evaluate whether diallyl sulfide could be a useful chemopreventive agent to reduce 3-methylcholanthrene-induced lung tumori-genesis in mice.

Materials and Methods

Materials

[α-^{32}P]dCTP (3,000 Ci/mmol) and [α-^{32}P]dUTP (3,000 Ci/mmol) were obtained from Amersham (Bucking-

hamshire, U.K.). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Animals

Male ICR mouse weighing 30-35 g were housed in polycarbonate cages and had free access to standard laboratory mouse chow and water. 3-methylcholanthrene animals were treated with four daily intraperitoneal injections of 3-methylcholanthrene in corn oil (25 mg/kg). 3-methylcholanthrene plus diallyl sulfide animals were treated with four daily intraperitoneal injections of 3-methylcholanthrene and a single intragastrical injection of diallyl sulfide (200 mg/kg). Control animals were injected with corn oil. Animals were sacrificed by cervical dislocation at 18 h after the last treatment.

Microsome preparation

Rat liver and lung microsomes were prepared by differential centrifugation (Hong *et al*., 1987). Protein concentration was determined by the method of Lowry *et al*. (1951).

7-Ethoxyresorufin-*O*-deethylase (EROD) activity

The dealkylation of ethoxyresorufin is sensitive and specific indicators of CYP 1A1 activity and was quantified by fluorimetric analysis of the accumulation of resorufin at an excitation wavelength of 522 nm and emission wavelength of 586 nm (Burke *et al*., 1974, 1985). Resorufin standards were assayed daily and utilized to calculate specific enzyme activity.

RNA isolation and hybridization analysis

Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). For Northern blot analysis, RNA was electrophoretically separated in 1% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filter. The nitrocellulose filter was baked at 80˚C for 2 h *in vacuo*. For hybridization with cDNA probe, the filter was prehybridized for 3 h at 55˚C in 5×Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 5× SSC, 0.1% SDS, 10 μg/ml of denatured salmon sperm DNA, and 50% formamide. The filter was then hybridized in the same solution with the ^{32}P-labeled cDNA probe (2×10^6 cpm/ml) overnight. The filter was washed at 65˚C twice with 1× SSC-0.5% SDS for 15 min each and then washed twice with 0.1× SSC-0.1% SDS for 15 min each. Autoradiography was carried out by exposing the filters to Kodak XAR-5 film at -70˚C with a Lightening plus intensifying screen. The bands on the autoradiographies were quantified a LKB densitometer.

cDNA synthesis and RT-PCR

Total RNA was subjected to reverse transcription (RT) and was then used in PCR experiments to generate partial cDNA probes for Northern blot analysis. Oligonucleotide primer set used to generate CYP1A1 cDNA probes were 5'-CCATGACCAGGAACTATGGG-3' and 5'-TCTGGTGAGCATCCAGGACA-3'. Reverse transcription of RNA was performed in a final volume of 20 μl containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM of each dNTP, 20 units of RNase inhibitor, 15 units AMV reverse transcriptase, 0.5 μg of oligo$(dT)_{15}$ and 1 μg of total RNA. The samples were incubated at 42˚C for 15 min, and reverse transcriptase was inactivated by heating at 95˚C for 5 min. To these complementary DNA samples, a PCR master mix was added to adjust the final volume to 50 μl. For CYP1A1 analysis, the final concentration in the PCR reaction was 3 mM $MgCl_2$, 2.5 units *Taq* polymerase, and 10 pmol of forward and reverse primers. The reaction mixtures were heated at 95˚C for 5 min and immediately cycled 30 times through a 1 min denaturing step at 94˚C, a 1.5 min annealing step at 54˚C, and a 1 min elongation step at 72˚C. Following the final cycle, a 5 min elongation step at 72˚C was included. Aliquots of the PCR reaction were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized with ethidium bromide staining.

Nuclei preparation and run-on transcription assay

Nuclei were purified by sucrose gradient centrifugation (Schebler *et al*., 1983). In brief, lung tissues were homogenized in 10 vol. of 0.3 M sucrose in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM Hepes, pH 7.5). The homogenate was filtered through six layers of cheesecloth, layered over a 10 ml cushion of 30% sucrose in buffer A, and spun down at 4˚C for 10 min at 3,000 rpm using a Sorvall GSA 600 rotor. The crude nuclei were resuspended in 3.5 ml of 2 M sucrose in buffer B (same as buffer A, except that the EGTA and EDTA concentrations were reduced to 0.1 mM), layered over 2 M sucrose in buffer B, and sedimented at 36,000 rpm in a Beckman SW 50.1 rotor at 4˚C for 1 h. The clean nuclei were resuspended in the nuclei storage buffer (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, 50% glycerol), sedimented for 2 min in a Eppendorf centrifuge at 4˚C, and resuspended in the nuclei storage buffer at a density of about 10^6 nuclei per microliter. The nuclei was checked under a microscope. Nuclei were either used immediately for the *in vitro* transcription assay or stored at -70˚C for

several weeks without loss of activity. The nuclear run-on transcription assay procedure was done as described by Zelenka *et al*. (1989). In brief, 5×10^6 nuclei were incubated in 100 μl of reaction mixture containing 25% glycerol, 5.8 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 173 mM KCl, 0.3 mM each ATP, GTP, and CTP, and 100 μCi of [α-^{32}P]UTP for 30 min at 30˚C. The reaction mixtures were then treated with DNase I (0.25 unit/μl, RNase free) for 10 min at 30˚C and digested with proteinase K (0.5 μg/μl). After the extraction with phenol and chloroform, carrier yeast tRNA was added (100 μg/sample) and the RNA solution was passed over a Nick column (Pharmacia, Piscataway, NJ) to remove the unincorporated [α-^{32}P]UTP. RNA was then precipitated with 0.4 M sodium acetate and 2.5 vol. of ethanol. The precipitate was dissolved in 100 μl of 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and digested with DNase I and proteinase K and extracted as described above. For complete removal of the unincorporated [α-^{32}P]UTP, the sample was further purified by passing over a second Nick column and the RNA was precipitated. The RNA pellet obtained from the final ethanol precipitation step was dissolved in 200 μl of 20 mM TES buffer (pH 7.4) containig 10 mM EDTA and 0.2% SDS. The ^{32}P-labeled RNA samples with equal amounts of radioactivities were then applied to nylon strips containing previously immobilized DNA probe. The volume of RNA solution used was sufficient to soak the nylon strip, usually 1.5 ml in a 5 ml scintillation vial. Hybridization was carried out 65˚C for 36 h. After hybridization, the strips were washed at 65˚C for 2 h in 2×SSC with several changes of the wash solution, blotted dry and autoradiographed for 4 to 7 days at -70˚C with Hyperfilm and an intensifying screen. The bands on the autoradiographies were quantified by using Bio-imaging analyzer system (BAS 2500, Fuji Photofilm Co.). The probe immobilized on the nylon filters for the hybridization included rat CYP1A1. The cDNA probe was denatured at 100˚C for 5 min, rapidly cooled in ice, and loaded onto nylon filters using a slot blot apparatus. Filters were prewet with H_2O and 6×SSC, then loaded per slot with 1 μg of gel-purified cDNA insert. Filters were baked at 80˚C for 2 h, and trimmed close to the DNA slots prior to hybridization.

Results

Effects of diallyl sulfide on 3-methylcholanthrene-induced EROD activity

To analyze the effects of diallyl sulfide on 3-methylcholanthrene-induced EROD activity, which is specific indicators of cytochrome P450 1A1 family, in mouse liver and lung, mice were treated with four injections of 3-methylcholanthrene (25 mg/kg, i.p.). EROD activity was induced by 3-methylcholanthrene treatment. EROD activity in mice treated with 3-methylcholanthrene plus diallyl sulfide was significantly lower than those in 3-methylcholanthrene-treated mice. diallyl sulfide itself slightly decreased and increased EROD activity in mouse liver and lung, respectively. Therefore, Diallyl sulfide could decrease 3-methylcholanthrene-induced EROD activity in mouse liver and lung (Table 1).

Effects of diallyl sulfide on 3-methylcholanthrene-induced levels of CYP1A1 mRNA

The effect of diallyl sulfide on 3-methylcholanthrene-induced CYP1A1 mRNA was investigated in mouse liver and lung. CYP1A1 mRNA levels were increased by 3-methylcholanthrene treatment. After 3-methylcholan-threne plus diallyl sulfide treatment,

Table 1. The effect of diallyl sulfide on EROD activity in 3-methylcholanthrene-treated mouse tissue microsomes. Each value represents mean ± S.D (N = 4).

Treatment	Liver (nmol/mg protein/min)		Lung (nmol/mg protein/min)	
Control	0.261	0.035	0.228	0.023
3-Methylcholanthrene	2.740	0.222[a]	0.462	0.068[a]
Diallyl sulfide	0.218	0.039[b]	0.265	0.015[b]
3-Methylcholanthrene + Diallyl sulfide	0.553	0.092[a,c]	0.256	0.042[a,d]

[a] significantly different from control value, $P < 0.001$

[b] significantly different from control value, $P < 0.05$

[c] significantly different from MC value, $P < 0.001$

[d] significantly different from MC value, $P < 0.05$

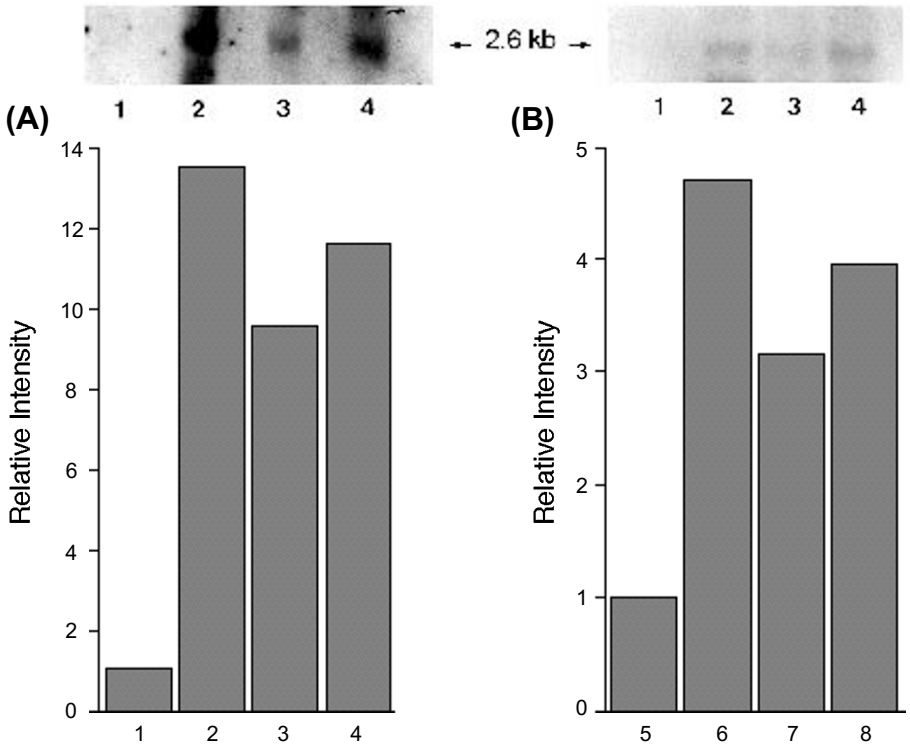

Figure 1 Northern blot analysis for expression of CYP1A1 mRNA. RNA was extracted from liver (A) and lung (B) tissues from 3-methylcholan-threne and 3-methylcholanthrene plus diallyl sulfide treated mice. RNA (20 μg) was separated in a 1% agarose with 2.2 M formaldehyde gel, transferred to nylon membrane and hybridized with ^{32}P-labeled cDNA. Lanes 1 and 5 , corn oil-treated; lanes 2 and 6 , 3-methylcholanthrene-treated; lanes 3 and 7, diallyl sulfide-treated; lanes 4 and 8, 3-methylcholanthrene + diallyl sulfide-treated.

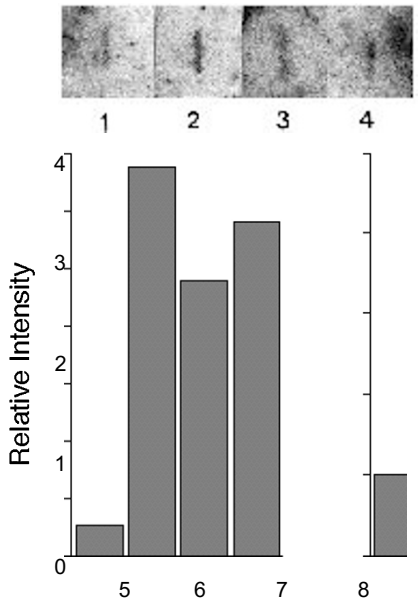

Figure 2 Nuclear run-on assay of lung nuclei. Nuclei were isolated from lung of untreated mice and mice treated with 3-methylcholanthrene and 3-methylcholanthrene + diallyl sulfide. Nuclear run-on assay was performed as described in method and transcripts hybridized to CYP1A1 cDNA. Lane 1, corn oil-treated; lane 2, 3-methylcholanthrene-treated; lane 3, diallyl sulfide-treated; lane 4, 3-methylcholanthrene + diallyl sulfide-treated.

CYP1A1 mRNA levels were significantly lower than those in 3-methylcholanthrene-treated mice (Figure 1). diallyl sulfide itself increased CYP1A1 mRNA levels. Therefore, in parallel with its inhibitory effects on 3-methylcholanthrene-induced EROD activity, diallyl sulfide could decrease 3-methylcholanthrene-induced CYP1A1 mRNA levels in mouse liver and lung.

In addition, induction of CYP1A1 mRNA by 3-methylcholanthrene treatment and decrease of 3-methylcholanthrene-induced CYP1A1 mRNA by 3-methylcholanthrene plus diallyl sulfide treatment were regulated at the transcription level according to the nuclear run on assay (Figure 2).

Discussion

A diet rich in fresh fruits and vegetables has been linked in epidemiologic studies to reduced risk for various forms of human cancer, most notably colorectal cancer (National Academy of Sciences, 1982). Supporting this relationship is the recent discovery of naturally occurring chemicals in certain vegetables and fruits which inhibit the process of carcinogenesis in animals (Fiala *et al*., 1985). However exciting as this is, many of these newly identified dietary inhibitors of cancer have been only selectively active in interfering with induction of cancer or mutagenesis induced by

polycyclic aromatic hydrocarbons (Newmark, 1984; Sparnins and Wattenberg, 1981). The need to discover chemical inhibitors of other structurally diverse yet equally potent carcinogens prompted this investigation of the thioethers in garlic. Members of *Allium* family are excellent sources of organic sulfides (Fenwick and Hanley, 1985). A long anecdotal history of the curative properties of herbs in this family has suggested that some pharmacologically active compounds could be present (Block, 1985). Investigation of organic sulfides for anticarcinogenic or chemopreventive properties was first attempted by Belman (1983) and more recently by Sparnins *et al*. 1986a, b). Sparnins *et al*. (1986a) have shown that allyl methyltrisulfide, one of the principal organic sulfides in garlic, maximally stimulated glutathione *S*-transferase activity in the A/J mice, which could account for significant inhibition of benzo(a) pyrene forestomach carcinogenesis. In another study these investigators found diallyl sulfide as well to be an effective inhibitor of lung cancer in mice (Sparmins *et al*., 1986).

Hepatic and extrahepatic total CYP content and CYP-dependent drug metabolism are known to decrease in animals subjected to various immunostimulatory conditions (Mannering and Deloria, 1986; Renton and Knickel, 1990). Proposed mechanisms for this effect include destruction of CYP enzymes by free radicals (Ghezzi *et al*., 1985), reduced CYP mRNA translation (Gooderham and Mannering, 1986; Renton and Knickel, 1990), and reduced CYP mRNA levels (Renton and Knickel, 1990). In the present study, diallyl sulfide itself slightly decreased and increased EROD activity in mouse liver and lung, respectively, and increased CYP1A1 mRNA level. In contrast to our findings, a recent study by Harber *et al*. (1995) revealed that diallyl sulfide enhanced rat hepatic microsomal EROD activity. In other work (Dragnev *et al*., 1995), diallyl sulfide exhibits minimal effects on the levels of CYP1A subfamily in rats. However, it is difficult to compare these findings with those in this study, because of different strains of animals used. In addition, we demonstrated that the induction of CYP1A1 mRNA and EROD activity by polycyclic aromatic compounds such as 3-methylcholanthrene is greatly reduced in mouse lung co-treated with diallyl sulfide. The modulation of carcinogen-metabolizing CYP expression by diallyl sulfide may have relevance to the process of chemical carcinogenesis and may contribute to individual variations in cancer susceptibility. Diallyl sulfide, by modifying CYP1A1 expression, may modulate the ability of this CYP to activate and/or detoxify carcinogens. There is an evidence that correlates increased inducibility of CYP1A1 activity with increased susceptibility to some forms of cancer in man (Kellermann *et al*., 1973; Guirgis *et al*., 1976; Trell *et al*., 1976; Kouri *et al*., 1982). Genetic, environmental and behavioral factors are known to influence individual susceptibility to some forms of chemically induced cancer. For example, cigarette smokers are known to have chronic lung inflammation (Linden *et al*., 1989). The degree of inflammation, however, varies from individual to individual (Linden *et al*., 1989), and therefore could result in differences among individuals in lung CYP1A1 expression and thus, carcinogen activation and/or detoxification.

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