Effect of Diallyl Sulfide on Rat Liver Microsomal Nitrosoamine Metabolism and Other Monoxygenase Activities

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ABSTRACT

It has been reported that p.o. administration of diallyl sulfide (DAS), a naturally occurring component of garlic (*Allium sativum*), inhibits 1,2-dimethylhydrazine-induced colon and liver cancer in rodents. A possible mechanism for this protective effect is inhibition of hepatic activation of the procarcinogen. The effect of DAS on P450IIE1, an isozyme of cytochrome P-450 which is active in the oxidative metabolism of dimethylhydrazine, was conveniently assayed in the present study by determination of N-dimethylhydrazine demethylase (NDMAd) activity at 1 min N-dimethylhydrazine in Sprague-Dawley rat liver microsomal incubations. DAS was found to be a competitive inhibitor of NDMAd, in contrast to the irreversible inactivation of NDMAd produced by carbon tetrachloride incubated under similar conditions. The inhibition by DAS of the demethylation of several substrates was selective. The thioether was most potent against N-dimethylhydrazine, less effective against N-nitrosomethylbenzylamine, and essentially ineffective against benzphetamine and ethylmorphine. Microsomes prepared at 3 h after DAS administration (200 mg/kg in corn oil intragastrically) showed moderate inhibition (<30% inhibition compared to control microsomes) of several monooxygenase activities; however, microsomes prepared 18 h posttreatment showed a marked decrease (about 80% inhibition compared to controls) in NDMAd activity, minor effects on other demethylase activities, and a 6-fold increase in pentoxyresorufin dealkylation. These trends at 18 h agreed with immunoblot analyses which showed suppression in the level of P450IIE1 and an elevation in P450IIB1. The selective inhibition of P450IIIE1 activity and suppression of its level in microsomes may contribute to the reported chemoprotective effects of DAS.

INTRODUCTION

The identification and exploitation of dietary anticarcinogens may substantially contribute to the prevention of cancer in humans. The salutary properties of garlic (*Allium sativum*), a widely consumed herb, have long been recognized in folklore (3) and are a current subject of scientific investigation (4-7). Diallyl sulfide [(CH2=CHCH2)2S], a component of garlic oil, has recently been shown to possess potent inhibitory activity toward the induction of colon (8, 9) and liver (10) cancer by 1,2-dimethylhydrazine (CH3NHNHCCH3) and toward esophageal cancer induced by NMBA (8-10) in laboratory animals. The bio-activation of dimethylhydrazine involves the sequential hepatic oxidation of azomethane (CH3N=NCH3) to azoxymethane [CH3N=NC(O)CH3] to methylazoxymethanol (HOCH3N=NC(O)CH3) (11). Since the virtual absence of this last catalytic activity in colon mucosa has been demonstrated (11) and probable routes for the transport and exposure of the reactive metabolites to the colon exist (11, 12), a potential mechanism for the anticarcinogenic property of DAS is inhibition of the initial hepatic conversions. Other sulfur-containing compounds have been shown to block azomethane N-oxidation *in vivo* (13).

As recently demonstrated using microsomal (14) and purified enzyme systems, oxidation of azoxymethane to methylazoxymethanol is catalyzed by cytochrome P450IIIE1. This isozyme is present constitutively in mammalian species (15-17) and is inducible by acetone or isoniazid treatment, fasting, diabetes, and numerous other factors (see Ref. 18). It is active in the metabolism of various small organic compounds such as NDMA, acetone, ethanol, carbon tetrachloride, and n-pentane. An interaction between P450IIIE1 and DAS was considered likely since this compound bears structural similarity to diethyl ether, an inhibitor of P450IIIE1-mediated reactions *in vivo* and *in vitro* and a high-affinity substrate for this isozyme (see Ref. 19).

In order to elucidate the mechanism for its anticarcinogenic properties, the effect of DAS on selected rat hepatic microsomal monooxygenase parameters was determined.

MATERIALS AND METHODS

Materials. Diallyl sulfide was obtained from Aldrich Chemical Co. (Milwaukee, WI). Corn oil was a product of Mazola, CPC International, Inc. (Englewood Cliffs, NJ). N-Nitrosomethylbenzylamine (NMBA) was from Ash Stevens, Inc. (Detroit, MI). p-Nitroanisole was from the Eastman Kodak Co. (Rochester, NY). Aminopyrine and ethylmorphine were obtained from Sterling-Winthrop Research Institute (Rensselaer, NJ) and Mallinckrodt Chemical Works (St. Louis, MO), respectively. Other chemicals were obtained from sources indicated previously (19).

Animals and Microsomes. Male Sprague-Dawley rats (90-100 g initial body weight) were maintained as described (19). The acetone and phenobarbital treatments were conducted using previous methods (19). Diallyl sulfide was given intragastrically at a dose of 200 mg/kg body weight in corn oil (1 ml/100 g body weight). Animals were sacrificed between 9 and 10 a.m. for preparation of microsomes (18).

Metabolic Assays and Analyses. The methods for determination of P-450 concentration, NADPH/P-450 reductase activity, and immunoblot analysis have been described (19). A previous method (18) for the determination of microsomal demethylase activity was used. The incubation volume was 0.5 ml unless otherwise indicated. DAS apparently dissolves in water at a concentration of 2 mM. For the inhibition studies, DAS was added from a fresh aqueous solution containing a 10-fold molar excess over the NDMAd substrate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. Supported by NIH Grants ES-03938 and CA-37137.

RESULTS

In Vitro Inhibition of Microsomal Monoxygenase Activities.

DAS was a potent inhibitor of the NDMA demethylase activity...
displayed by acetone-induced rat liver microsomes. In incubations containing varied concentrations of substrate, an apparent \( K_m \) of 25.8 ± 2.3 (SD) \( \mu \text{M} \) (\( n = 3 \)) was observed. DAS exhibited competitive inhibition with an apparent \( K_i \) of 26.8 ± 7.2 \( \mu \text{M} \) (\( n = 3 \)) (Fig. 1).

In order to assess the selectivity of the inhibitory actions of DAS, its effect on monoxygenase activities associated with control and phenobarbital-induced microsomes was determined (Table 1). In both types of microsomes, DAS was a potent inhibitor of NDMA demethylation, a moderate inhibitor of NMBA demethylation, and a weak inhibitor of benzphetamine and ethylmorphine demethylations. These results demonstrated that the inhibition by DAS was not a general effect on all mixed-function oxidase activities but was somewhat selective.

**Influence of DAS and Carbon Tetrachloride on NDMA Demethylation and on the Stability of Cytochrome P-450.** The possibility of a metabolism-dependent inactivation of NDMA demethylation by DAS was investigated by determination of the time dependency of the inhibition and by comparison with the effects of a previously characterized (20) irreversible inhibitor, carbon tetrachloride (Table 2). A 20-min preincubation, lacking inhibitors, in the absence or presence of NADPH resulted in a slight decrease in the NDMA demethylation determined in a subsequent 10-min incubation. Preincubation in the presence of either DAS or carbon tetrachloride, but in the absence of NADPH, resulted in a moderate reduction in the inhibition that had been observed without preincubation. The inhibition persisted when NADPH was present during the preincubation with carbon tetrachloride but was essentially eliminated when DAS was preincubated under similar conditions. These observations confirmed an irreversible inactivation by carbon tetrachloride and were consistent with the competitive inhibition by DAS described above. The possibility that DAS was converted to a noninhibitory metabolite during the preincubation was supported by a preliminary gas chromatographic analysis which revealed a >90% decrease in the concentration of DAS when NADPH was present (not shown).

This contrast between the two inhibitors was also apparent when their effects on the binding of CO to dithionite-reduced microsomes, after a 40-min incubation, were compared (Table 3). The decrease in CO binding during 40 min in the control was probably due to lipid peroxidation (21). The presence of DAS during the incubation had no effect on the CO binding compared to the control, while carbon tetrachloride gave a pronounced decrease in measurable P-450.

**Influence of DAS Treatment on Microsomal and Soluble Monoxygenase Activities.** In previous in vivo studies (8–10), DAS usually was administered intragastrically 1–3 h prior to exposure to prococarcinogen. The foregoing in vitro studies demonstrated that DAS acted as a competitive inhibitor of certain monoxygenase activities. The possibility that administered DAS p.o. also exerts either an inductive or suppressive effect on these activities was also examined. A slight inhibition of NDMA demethylase and other oxidative activities was observed in microsomes and in the postmitochondrial supernatant fraction (S-9) obtained 3 h after the treatment (Table 4). The percentage of inhibition in both the S-9 fraction and in microsomes was not diminished at higher NDMA concentrations. These observations were not consistent with competitive inh-
**DISCUSSION**

Previous studies (8–10) have demonstrated that DAS blocks the induction of carcinogenic responses in liver and colon which develop subsequent to dimethylhydrazine administration in rodents. The present findings suggest that a plausible mechanism is the selective inhibition of cytochrome P450IE1, which is involved in the initial hepatic activation of the procarcinogen (14). This isozyme activity is conveniently assayed in microsomes by monitoring the demethylation at 1 mM NDMA (18).

Two modes of inhibition by DAS of NDMA demethylase activity were evident. The first type, observed in incubations using acetone-induced microsomes, was competitive inhibition. The low apparent affinity suggested that DAS has a high affinity for P450IE1 which would be in agreement with previous studies using structurally analogous compounds such as diethyl ether (Km 13 μM) and pentane (Km 9 μM) (see Ref. 19). The presence of a small hydrophobic binding pocket at the active site has been proposed7 and would accommodate DAS and similar compounds. As expected, the observed inhibition was selective toward NDMA metabolism, as compared with the oxidation of substrates catalyzed by other P-450 isozymes (Table 1).

The possibility of irreversible inactivation was considered since substrates containing carbon-carbon double bonds are often involved in the destruction of P-450 (23). Furthermore, a potential product of the P-450-dependent oxidation of a compound such as DAS is the sulfhydryl-reactive reagent, acrolein (OCH2CH=CH2) (23, 24). However, no evidence for an irreversible inhibition by DAS was observed. The effect of varying the concentration of DAS was not determined but could influence the observed inhibition (24). Preliminary experiments using gas chromatographic analysis showed that initial levels of DAS decreased in incubations containing NADPH. Two

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**Table 4** Effect of 3-h or 18-h corn oil (C.O.) or DAS pretreatment on microsomal and S-9 fraction monooxygenase activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NDMA (1 mM)</th>
<th>NDMA (0.2 mM)</th>
<th>N MBA</th>
<th>Benzphetamine</th>
<th>Ethylmorphine</th>
<th>p-Nitroanisole</th>
<th>Aminopyrine</th>
<th>7-Pentoxyresorufin O-dealkylation* (pmol resorufin/min/mg)</th>
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<tbody>
<tr>
<td>Microsomes</td>
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<tr>
<td>C.O. (3 h)</td>
<td>2.31 ± 0.73</td>
<td>2.07 ± 0.66</td>
<td>1.14 ±0.26</td>
<td>6.27 ± 1.01</td>
<td>8.40 ± 1.01</td>
<td>2.78 ± 0.88</td>
<td>3.93 ± 1.22</td>
<td>20.6 ± 2.8</td>
</tr>
<tr>
<td>DAS (3 h)</td>
<td>1.72 ± 0.24</td>
<td>1.48 ± 0.21</td>
<td>0.98 ±0.14</td>
<td>5.60 ± 1.08</td>
<td>7.45 ± 1.61</td>
<td>1.98 ± 0.27</td>
<td>3.12 ± 0.83</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>C.O. (18 h)</td>
<td>3.30 ± 0.28</td>
<td>2.92 ± 0.25</td>
<td>1.53 ±0.16</td>
<td>7.35 ± 1.06</td>
<td>12.79 ± 1.31</td>
<td>3.56 ± 0.03</td>
<td>5.11 ± 0.43</td>
<td>12.5 ± 1.5</td>
</tr>
<tr>
<td>DAS (18 h)</td>
<td>0.71 ± 0.041</td>
<td>0.53 ± 0.022</td>
<td>1.06 ±0.16</td>
<td>8.77 ± 0.63</td>
<td>5.76 ± 0.47</td>
<td>2.08 ± 0.07</td>
<td>3.97 ± 0.17</td>
<td>74.3 ± 21.1</td>
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<tr>
<td>S-9 fraction</td>
<td></td>
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<tr>
<td>C.O. (3 h)</td>
<td>0.47 ± 0.17</td>
<td>0.34 ± 0.16</td>
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<tr>
<td>DAS (3 h)</td>
<td>0.23 ± 0.05</td>
<td>0.20 ± 0.06</td>
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* Substrates were present at 1 mM unless otherwise indicated. Values are the mean ± SD from four separate microsomal preparations.

* For comparison, the rate by microsomes from phenobarbital-induced rats was 1350 pmol resorufin/min/mg. The substrate concentration was 10 μM.

Values are significantly different from those of the corresponding control, P < 0.002.

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Fig. 2. Immunoblot analysis of hepatic microsomes from control and diallyl sulfide-treated rats. Microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot analysis. Lanes a–e are immunoblots using anti-P450IE1 IgG; Lanes f–j are immunoblots using anti-P450IE1 IgG. Lanes b and c (also corresponding to Lanes g and h, respectively) contained microsomal proteins from two separate control rats. Lanes d and e (also corresponding to Lanes i and j, respectively) contained microsomal proteins from two separate diallyl sulfide-treated rats. Purified P450IE1 (0.13 μg) was applied to Lane A. Lane h–j contained 5.3 μg microsomal protein each.
microsomal enzyme systems, a flavin-containing monoxygenase and P-450, oxidize sulfur-containing compounds (25–27). Determination of the products and enzyme specificity of DAS metabolism will require further investigation.

A second mode of inhibition of NDMA demethylase was observed after DAS administration in vivo. The selective suppression in the activity and in the immunologically determined levels of P450IIIE1 could substantially contribute to the reported chemoprotective properties of DAS. The inactivation was time dependent, suggesting a requirement for metabolism and redistribution of this metabolite. The in vivo metabolic fate of the compound is unknown but may be examined by using radiolabeled substrate (28). The decrease in the level of P450IIIE1 could also be due to an inhibition in the production of this isozyme.

DAS potentially affects monoxygenase activities in nonhepatic tissues. The reported elimination of NMBA-induced DNA alkylation by P450 content, supported the notion that DAS is a specific inhibitor of microsomal isozymes of P-450 present in the mucosa that catalyze the activation of NMBA to alkylating agents (29, 30).

The increase of P450IIIE1 observed in the present in vivo study was unexpected, but along with the minimal change observed in P-450 content, supported the notion that DAS is not a general suppressor of monoxygenase activities. This level of elevation of P450IIIE1 was also observed after administration of diethyl ether (19). The mechanism of induction by these, and by the potent prototypic agent phenobarbital, is not known but may involve hormonal changes (31).

Doses of DAS used previously and in the present study were quite high (up to 200 mg/kg body weight) considering the estimate that <1 mg is present in a clove of garlic (9). The octanol:water partition ratio for DAS has not been reported but is 89 for the structurally similar compound diethyl sulfide (32). DAS might partition into lipid-rich environments such as microsomal membranes, increasing its local concentration and prolonging its effect. Thus moderate levels of DAS consumed in a normal diet may nonetheless inhibit the activation of low levels of some types of environmentally encountered procarcinogens.

Further understanding of the biochemical properties of DAS and related compounds will aid in the search for other naturally occurring and synthetic anticarcinogenic agents.

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