Diallyl sulfide inhibits the oxidation and reduction reactions of stilbene estrogens catalyzed by microsomes, mitochondria and nuclei isolated from breast tissue of female ACI rats

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Previously, it has been demonstrated that microsomal, mitochondrial and nuclear enzymes isolated from the liver of male Sprague-Dawley rats catalyzed the oxidation of diethylstilbestrol (DES) to DES quinone. In the present study we have shown that diallyl sulfide (DAS) inhibits the oxidation of DES to DES quinone in all three subcellular fractions (microsomes, mitochondria and nuclei) isolated from breast tissue of female ACI rats. UV analysis of mitochondrial and microsomal fractions revealed that DAS decreased the rate of DES oxidation to DES quinone and DAS also decreased the rate in which DES quinone was reduced to DES. Lineweaver–Burk plots of the rate of DES quinone formation at various DES and DAS concentrations demonstrated that DAS inhibited the oxidation of DES and the reduction of DES quinone in a non-competitive fashion. In both microsomal and mitochondrial oxidation reactions the K_m remained constant whereas the V_max decreased with increasing DAS (0, 186 and 373 μM) concentrations (microsomes K_m = 80 μM; V_max = 5.56, 4.16 and 3.33 nmol/mg protein/min; mitochondria K_m = 35.7 μM; V_max = 3.45, 2.44 and 1.82 nmol/mg protein/min). Results were similar for reduction reactions. HPLC analysis revealed that a concentration of 186 μM DAS inhibited the mitochondrial, microsomal and nuclear oxidation by 27, 35 and 40%, respectively. A concentration of 373 μM DAS inhibited the mitochondrial, microsomal and nuclear oxidation by 50, 52 and 60% respectively. The data provide direct evidence that the breast tissue contains the metabolic machinery required to oxidize DES to reactive intermediates that may lead to genetic instability and cancer. This inhibition may play a role in the chemoprevention of stilbene estrogen-induced breast cancer.

Introduction

It has been shown that estrogens including diethylstilbestrol (stilbene estrogen) are metabolized to reactive intermediates, such as semiquinone and quinone (1,2). Microsomal, mitochondrial and nuclear enzymes are all involved in the oxidation and reduction of DES (3,4). The reactive metabolites of diethylstilbestrol have been shown to covalently bind to DNA (5) and nuclear proteins (6). The metabolism of estrogen is believed to play a major role in estrogen-induced cancer (7).

Several sulfur containing compounds with chemopreventive properties have been isolated from garlic, the most effective one being diallyl sulfide (DAS) (8). Diallyl sulfide has been found to inhibit dimethylhydrazine-induced colon carcinoma in female C57NL/6 J mice (9), N-nitrosodimethylamine induced esophageal carcinoma in male Sprague–Dawley rats (10), N-nitrosodiethylamine induced pulmonary adenoma in female A/J mice (11), benzo[a]pyrene induced forestomach tumor and pulmonary adenoma in female A/J mice (12) and hepatocarcinogenesis induced by 1,2-dimethylhydrazine in Fisher 344 rats (13). It has been shown that DAS inhibits the production of DES-induced DNA adducts presumably via metabolic modulation (14). Whether or not DAS will inhibit estrogen induced cancer or estrogen metabolism is yet to be determined.

In the present study the inhibition of microsomal, mitochondrial and nuclear oxidation of DES to DES quinone by DAS has been investigated. This study provides direct evidence for the inhibition of the oxidation and reduction of DES by DAS in various organelles. DES reactive intermediates generated during oxidation are able to covalently bind to both mitochondrial and nuclear DNA (15). Analogous in vivo oxidation of DES to reactive metabolites and covalent modifications in DNA by reactive products may be factors in DES-induced cancer. Furthermore, the inhibition of the production of these reactive metabolites may prevent DES-induced cancer.

Materials and methods

Chemicals

NADH, diethylstilbestrol, phenylmethylsulfonyl fluoride (PMSF), cumene hydroperoxide, β-naphthoflavone, digitonin and diallyl sulfide were purchased from Sigma Chemical Co., St Louis, MO. Female ACI rats 7–8 weeks old were purchased from Harlan, Indianapolis, IN.

Animal treatment

Ten female ACI rats were treated for 4 days with a daily dose of β-naphthoflavone (50 mg/kg i.p.) to induce the IA family of cytochrome p-450 (2). The rats were killed by carbon dioxide exposure. The breast tissue was removed, weighed and homogenized in 1:10 wt/vol (0.25 M sucrose, 1.0 mM EDTA, 2.5 mM PMSF). Microsomes, mitochondria and nuclei from the breast tissue were isolated by differential centrifugation. The organelles were used to catalyze the oxidation and reduction reactions of DES. The oxidation and reduction products generated by the organelles were analyzed by UV absorption and HPLC analysis.

Preparation of nuclei

Nuclei were isolated according to the procedure of Rickwood et al. (16). In brief, tissue was homogenized in 2 vol of 0.25 M sucrose solution containing 1.0 mM PMSF. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 1000 g. The pellet obtained was suspended in 0.25 M sucrose and 4 vol of 2.3 M sucrose was under-laid. The nuclei were collected by centrifugation at 100 000 g for 60 min. The nuclei were then washed with 0.25 M sucrose containing 0.2 mM PMSF. The purity of nuclei was checked after staining with hematxylin by phase contrast microscopy. Glucose 6-phosphate and cytochrome C oxidase activity was assayed to determine microsomal and/or mitochondrial contamination (17,18). It was determined

Abbreviations:

DAS, diallyl sulfide; DES, diethylstilbestrol; PMSF, phenylmethylsulfonyl fluoride.
that there was <1% contamination of both microsomes and mitochondria based on enzymatic activity.

Microsomal preparation
Breast tissue homogenate was centrifuged at 12 000 g for 30 min to eliminate nuclei and mitochondria. The supernatant was centrifuged at 120 000 g to collect microsomes. The purity of the microsomes was assessed by both morphological and biochemical analyses. Microsomes were stained with eosin and hematoxylin. Phase contrast microscopy did not reveal any nuclear contamination. The determination of cytochrome C oxidase (17) demonstrated <1% mitochondrial contamination.

Mitochondrial preparation
Breast tissue was centrifuged at 3000 g for 30 min to remove nuclei and cellular debris. The supernatant was centrifuged at 11 000 g to collect mitochondria. The mitochondria was treated with 1.6% digitonin to remove the outer membrane and cellular contamination. The mitoplasts (mitochondria without outer membrane) was collected by centrifugation for 30 min at 11 000 g for 30 min (19). The purity of mitoplasts was assessed by both morphological and biochemical analyses. Mitoplasts were stained with eosin and hematoxylin. Phase contrast microscopy did not reveal any cellular contamination. The determination of cytochrome C oxidase activity (17), an enzymatic marker of mitochondria, showed 100-110 nmol/mg protein/min specific activity. Microsomal contamination was assessed by measuring the activity of glucose 6-phosphatase, an enzymatic marker of endoplasmic reticulum (18). The activity of glucose 6-phosphatase was 601 pmol/mg protein/min in microsomes. However, the activity of glucose 6-phosphatase was 21 pmol/mg protein/min in our mitochondrial preparations. These values demonstrate that our mitoplasts were free of nuclei and microsomes. This is in agreement with the report of Niranjan et al. (20,21).

Oxidation reaction system
The reaction condition for the conversion of DES to DES quinone as described by Roy and Liehr (2) was used. The reaction condition consists of 120 μM cumene hydroperoxide, 420 μg/ml mitoplasts or 346 μg/ml of microsomes, in a final volume of 1 ml of 10 mM phosphate buffer, pH 7.5. Various concentrations (0-120 μM) of DES were used to determine the kinetic constants of the reactions. Various concentrations of DAS (186 and 373 μM) were added to determine the type of inhibition that DAS has on the oxidation of DES. No cumene hydroperoxide (oxidation cofactor) was used in control reactions. The conversion of DES quinone to DES was monitored as a gradual decrease in UV absorption in the range of 280–400 nm. The formation of reduction products was analyzed by HPLC (2). DES quinone and its metabolites were extracted with ethyl ether.

HPLC analysis
The oxidation and reduction products were extracted with water saturated ethyl ether. The reaction mixture was dried under nitrogen and metabolites were reconstituted in methanol. An appropriate amount (10–50 μl) of sample was injected into the HPLC. A methanol/water gradient consisting of 36–83% methanol was run using a C14 reverse phase column from 0 to 30 min at a flow rate of 1 ml/min. The UV detection was performed at a wavelength of 254 nm. The statistical significance was determined using a one-way ANOVA on SAS statistical software.

Results
Inhibition of DES oxidation by DAS
Mitoplasts were incubated in the presence of DES and cumene hydroperoxide using the oxidation reaction system described in the methods. DES quinone was detected by UV spectroscopy. The UV spectral analysis of the mitochondrial mixture containing DES and cumene hydroperoxide revealed a gradual increase in the absorbance at 312 nm. The spectral pattern was identical to that of synthetic DES quinone (Figure 1). In the control reactions, no DES quinone was produced. In oxidation reactions that contained DAS (373 μM) the production of DES quinone was reduced by 50%. The rate of DES quinone formation in the presence of mitoplasts and cumene hydroperoxide was dependent on the concentration of DES (Figure 2). A Lineweaver–Burk plot of rate of formation of DES quinone at various concentrations of DES yielded a $K_m$ of 35.7 μM and $V_{max}$ of 3.45 nmol/mg protein/min. The kinetic constants of the reactions were determined by using various concentrations of DES (0–120 μM) and two concentrations of DAS (186 and 373 μM). With increasing concentrations of DAS the $K_m$ remained constant whereas the $V_{max}$ decreased (3.45, 2.44 and 1.82 nmol/mg protein/min) (Figure 2). The data indicate that the mitochondria are capable of metabolizing DES quinone. No NADH (reduction cofactor) was used in control reactions. The conversion of DES quinone to DES was analyzed by HPLC (2). DES quinone and its metabolites were extracted with ethyl ether.

HPLC analysis
The oxidation and reduction products were extracted with water saturated ethyl ether. The reaction mixture was dried under nitrogen and metabolites were reconstituted in methanol. An appropriate amount (10–50 μl) of sample was injected into the HPLC. A methanol/water gradient consisting of 36–83% methanol was run using a C14 reverse phase column from 0 to 30 min at a flow rate of 1 ml/min. The UV detection was performed at a wavelength of 254 nm. The statistical significance was determined using a one-way ANOVA on SAS statistical software.
DES to DES quinone and DAS is capable of inhibiting this metabolism. Based on the kinetic constants the nature of this inhibition seems to be non-competitive. Results from the oxidation in microsomal reactions were similar to those in mitochondria. The microsomes were capable of oxidizing DES to DES quinone and DAS inhibited this oxidation (data not shown).

The kinetic constants for the mitochondrial and microsomal oxidation reactions are summarized in Table I. The kinetic constants were not determined for nuclear oxidation reactions due to the small amount of nuclei that could be isolated from the breast tissue.

### Table I. Kinetic constants of oxidation and reduction reactions

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<tr>
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<th>Oxidation</th>
<th>Reduction</th>
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<tr>
<td></td>
<td>Microsomes</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>$K_m$</td>
<td>80 $\mu$M</td>
<td>35.7 $\mu$M</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>5.56</td>
<td>3.45</td>
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<tr>
<td>0 $\mu$M DAS</td>
<td>4.16</td>
<td>2.44</td>
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<tr>
<td>186 $\mu$M DAS</td>
<td>3.33</td>
<td>1.82</td>
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<tr>
<td>373 $\mu$M DAS</td>
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The unit for $V_{max}$ is nmol/mg protein/min.

Fig. 2. Influence of various substrate concentrations of DES on the rate of oxidation of DES to DES quinone by mitoplasts: The reaction mixture consisted of mitoplasts (0.42 mg equivalent protein), 120 $\mu$M cumene hydroperoxide, and various concentrations of DES (0–100 $\mu$M) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. A Lineweaver–Burk plot of rate of formation of DES quinone and its inhibition by DAS revealed a constant $K_m$ with decreasing $V_{max}$. Values represent the means of four experiments.

**HPLC results**

HPLC analysis of the production of oxidation and reduction products of DES in microsomes, mitochondria and nuclei were similar to that demonstrated by UV analysis. DAS inhibited oxidation and reduction reactions of DES in a dose-dependent fashion. A concentration of 186 $\mu$M DAS inhibited the mitochondrial, microsomal and nuclear oxidation by 27, 35 and 40%, respectively (Figure 4). The data indicate that mitochondria are capable of reducing DES quinone to DES and DAS is capable of inhibiting this reduction. Based on the kinetic constants, the nature of this inhibition seems to be non-competitive. Results from the reduction in microsomal reactions were similar to those in the mitochondria. The microsomes were capable of reducing DES quinone to DES and DAS inhibited this reduction (data not shown).

The kinetic constants for the mitochondrial and microsomal reduction reactions are summarized in Table I. The kinetic constants were not determined for nuclear reduction reactions due to the small amount of nuclei that could be isolated from the breast tissue.

### Discussion

It is commonly recognized that the liver is the major organ of xenobiotic biotransformation. We have demonstrated for the first time that organelles (mitochondria, microsomes and nuclei) isolated from the breast of female ACI rats catalyze the oxidation and reduction reactions of DES. This is significant in that it provides evidence that breast tissue is capable of metabolizing estrogens into reactive products that can cause DNA damage. Redox-cycling of DES has been demonstrated to produce reactive oxygen species such as superoxide radicals.
and DES quinone (1). These reactive molecules can cause DNA damage and ultimately mutations that cause cancer. The demonstration of the redox-cycling of DES by mitoplasts and nuclei are of significance in that these are not traditional organelles of metabolic study. However, they contain the most critical macromolecule (DNA) in regards to carcinogenesis.

The role that the mitochondria play in carcinogenesis is not clear. However there are many correlations with alterations in the mitochondria and carcinogenesis. It has been shown that mitochondrial DNA is the primary site of attack by reactive...
metabolites of benzo[a]pyrene, aflatoxin B1, 7,12-dimethylbenz[a]anthracene and 3-methylcholanthrene (22–24). In the case of benzo[a]pyrene the adduct level in the mitochondria was 40 times higher than that found in genomic DNA and these adducts persist longer in mitochondrial DNA (21). The mitochondria of tumor cells are frequently structurally and functionally different from those isolated from normal cells (22–24). This evidence supports the idea that mitochondrial metabolism may play a crucial role in carcinogenesis. In addition to demonstrating that these organelles can metabolize DES to reactive intermediates, we have demonstrated that DAS inhibits this metabolism in a non-competitive fashion in mitochondria and microsomes. This inhibition may help explain the mechanism of the chemopreventive actions of DAS.

Furthermore, we have shown that DAS when given with DES, inhibits the formation of DES-induced DNA adducts in vitro and in vivo (14). This demonstrates that direct inhibition of DES metabolism plays a role in the inhibition of DES-induced DNA adduct formation. We have also shown that DAS administered several days prior to DES, inhibits the formation of DES-induced DNA adducts (14). This implies that some process in addition to the direct inhibition of metabolism by DAS is involved in the inhibition of DES-induced DNA adducts. It has been shown that DAS induces glutathione S-transferase, epoxide hydrolase and UDP-glucuronosyltransferase in the liver (25). We propose that the expression of these enzymes may play a crucial role in carcinogenesis. In addition to demonstrating that these organelles can metabolize DES to reactive intermediates, we have demonstrated that DAS inhibits this metabolism in a non-competitive fashion in mitochondria and microsomes. This inhibition may help explain the mechanism of the chemopreventive actions of DAS.

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References


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