Short Communication

FORMATION OF N-ALKYLPROTOPORPHRIN IX FROM METABOLISM OF DIALLYL SULFONE IN LUNG AND LIVER

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ABSTRACT:

Diallyl sulfone (DASO2) is a garlic derivative formed during cooking or after ingestion. Bioactivation of DASO2 in murine lung and liver results in formation of an epoxide that inactivates CYP2E1 and significantly decreases cytochrome P450 and heme levels. In this study, we tested the hypothesis that DASO2 metabolism leads to production of the heme adduct, N-alkylprotoporphyrin IX (N-alkyl-IPP). Formation of N-alkylIPP in vivo was determined by spectrophotometric and fluorometric methods, respectively. In in vivo studies, N-alkylIPP was generated in the livers of male and female mice treated with DASO2, but was not detectable in the lungs of DASO2-treated mice. In in vitro studies, rates of formation of N-alkylIPP in liver and lung microsomes incubated with DASO2 and NADPH were dependent on time and protein concentrations, but were negligible in control incubations performed in the absence of NADPH or DASO2 or with boiled microsomes. The rates of N-alkylIPP formation generated in murine liver were higher than those in either murine lung or human liver. Kinetic analysis revealed that murine liver microsomes metabolized DASO2 to N-alkylIPP with higher affinity and catalytic efficiency than did murine lung or human liver microsomes. Recombinant rat CYP2E1 also metabolized DASO2 to N-alkylIPP; however, rates of formation of the heme adduct was minimal in incubations of recombinant human CYP2E1 with DASO2. These findings demonstrated that the N-alkylIPP adduct was produced via metabolism of DASO2 in murine liver and lung microsomes, in human liver microsomes, in recombinant CYP2E1, and in vivo in murine liver.

In 1892, Semmler, a German chemist, applied steam distillation to cloves of garlic (Allium sativum) and produced a strong-smelling oil that on further purification produced diallyl sulfides (Block, 1985). The odoriferous constituent of garlic is allicin, which is formed by enzymatic conversion of 7-alliin to alliinase to allicin. Allicin is an unstable component that can be further transformed to other garlic compounds including diallyl sulfide (DAS). In addition to being a component of garlic oil, DAS can be produced during cooking or after ingestion of garlic (Hayes et al., 1987). It has been estimated that 1 g of garlic yields approximately 30 to 100 µg of DAS (Sparrins et al., 1988).

Previous studies have identified both diallyl sulfide (DASO) and diallyl sulfone (DASO2) in extracts of liver, blood, and urine from rats treated with DAS, suggesting that DASO and DASO2 are derived from DAS (Bradly et al., 1991b). Studies with rat liver microsomes indicated that DAS undergoes sequential metabolism to DASO and DASO2. Further studies confirmed that CYP2E1 catalyzes oxidation of the sulfur atom of DAS to yield DASO and subsequently DASO2 (Jin and Baillie, 1997). Although these garlic derivatives are all competitive inhibitors of CYP2E1, the inhibitory effect of DASO on CYP2E1 is more pronounced and is manifested more rapidly than by either DAS or DASO (Brady et al., 1991a). The efficacy of DASO2 as a CYP2E1 inhibitor has been ascribed to mechanism-based inactivation, and it is the metabolic event involving DASO2 that leads to CYP2E1 inactivation, which mediates the chemoprotective effects of DAS (Jin and Baillie, 1997).

Previous studies have investigated the mechanisms responsible for the inactivation of CYP2E1 and the protective effect of DASO2 against lung cytotoxicity induced by 1,1-dichloroethylene (Forkert et al., 1996a,b, 2000; Premdas et al., 2000). The results showed that DASO2 undergoes P450-dependent oxidation at one of its terminal double bonds to form diallyl sulfone monooxepoxide (1,2-epoxypropyl-3,3'-sulfonyl-1'-propene; DASO3) (Fig. 1), a reactive metabolite believed to be responsible for CYP2E1 inactivation. Levels of immunodetectable CYP2E1, total cytochrome P450, and heme were all reduced (Premdas et al., 2000). Incubation of murine liver microsomes with DASO2 (1.0 mM) decreased total cytochrome P450 and heme levels by about 30% and 70%, respectively. Immunodetectable CYP2E1 was reduced and correlated with a 70% decrease in p-nitrophenol hydroxylation 2 h after treatment of mice with DASO2 (100 mg/kg p.o.). These findings suggested the possibility that bioactivation of DASO2 produces DASO3, which alkylates the heme moiety at one of the four pyrrole nitrogens within the active site of P450, yielding the heme adduct N-alkylprotoporphyrin IX (N-alkylIPP; Fig. 2) (for review, see Ortiz de Montellano and Correia, 1983, 1995). Here, we have undertaken studies to test the hypothesis that metabolism of DASO2 leads to the formation of N-alkylIPP, an event that is likely associated with loss of heme and inactivation of cytochrome P450 reported previously (Premdas et al., 2000). Our results confirmed that the N-alkylIPP adduct is produced from oxidative metabolism of DASO2 in murine liver and lung microsomes, in human liver microsomes, in recombinant rat CYP2E1, and in vivo in murine liver.

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ABBREVIATIONS: AIA, allylisopropylacetamide; N-alkylIPP, N-alkylprotoporphyrin IX; N-ethylIPP, N-ethylprotoporphyrin IX; DAS, diallyl sulfide; DASO, diallyl sulfone; DASO2, diallyl sulfone; DASO3, 1,2-epoxypropyl-3,3'-sulfonyl-1'-propene.
Materials and Methods

Materials. Chemicals and reagents were obtained from suppliers as follows: potassium phosphate, NADPH, dichloromethane (high-performance liquid chromatography grade), sodium sulfate (anhydrous), zinc acetate, and iodoethane (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada); sulfuric acid (E. Merck, Stockholm, Sweden); protoporphyrin IX dimethyl ester (Frontier Scientific Porphyrin Products, Logan, UT); and Silica Gel G thin-layer chromatography plates (1000 and 2000 µm) (Analtech, Newark, DE). Diallyl sulfone was synthesized by Color Your Enzyme (Bath, ON, Canada). Human liver microsomes and recombinant rat and human CYP2E1 were obtained from BD Biosciences Discovery Labware (Bedford, MA). Allylisopropylacetamide (AlIA) was a donation from Dr. Gerald S. Marks (Department of Pharmacology and Toxicology, Queen’s University, Kingston, ON, Canada).

Animal Treatment. Male and female CD-1 mice, weighing 22 to 30 g, were obtained from Charles River Canada (St. Constant, QC, Canada). The mice were maintained on a 12-h light/dark cycle and were given free access to water and food (Mouse diet 5015; PMI Nutrition International Inc., Brentwood, MO). For the in vivo experiments, male and female mice were treated with 100 mg/kg DASO2 in water (p.o.); control mice were treated with water. After 2 h, the mice were anesthetized with sodium pentobarbital (120 mg/kg i.p.). For the in vivo studies, livers from 3 mice and lungs from 10 mice were pooled for each sample. Tissues were homogenized in ice-cold H2SO4 in methanol (5% v/v; 10–15 ml/g tissue). The homogenates were stored in the dark at 4°C for 24 h, after which purification and isolation of N-alkylPP was performed according to a method described previously (Wong et al., 1998). Briefly, the N-alkylPP dimethyl ester was isolated from the homogenates, reacted with zinc acetate (25 µmol) in methanol to form the zinc-complexed N-alkylPP dimethyl ester, and dried. The residue was dissolved in 2 ml of dichloromethane, applied to a Silica Gel G thin-layer chromatography plate (2000 µm), and developed in dichloromethane/methanol (260:39 v/v) for 60 min. The single green band (Rf = 0.68–0.74) (Wong and Marks, 1999) that exhibited red fluorescence (corresponding to reacted dimethyl esters) under long-wave ultraviolet light was scraped off the plate, extracted with acetone, and evaporated to dryness. The residue was then dissolved in 2 ml of methanol containing zinc acetate (25 µmol) and dried using a rotary evaporator. This final residue was dissolved in 2 ml of dichloromethane, and the absorption spectrum of the sample was determined by scanning from 400 to 800 nm, using a Beckman Coulter (Fullerton, CA) DU 640B spectrophotometer. The concentration of Zn-N-alkylprotoporphyrin IX was estimated using the molar extinction coefficient (ε > 128,000 m⁻¹ cm⁻¹ at 432 nm) for the Zn-N-ethylprotoporphyrin IX (N-ethylPP) dimethyl ester (Ortiz de Montellano and Mico, 1981).

Synthesis of Zn-N-ethylPP Dimethyl Ester. The adduct N-ethylPP has been used in previous studies as an analog for other alkylated heme adducts (Lavigne et al., 2002). The method of De Matteis et al. (1980) was used to synthesize N-ethylPP. Protoporphyrin-IX dimethyl ester (3.2 mg) was reacted with iodoethane (2 ml) for 18 h at 105°C in a sealed 5-ml test tube. The residue was purified by thin-layer chromatography using a silica plate (2000 µm) as described previously (Kimmert and Marks, 1992). The reacted dimethyl ester was extracted with acetone, dried, and resuspended in methanol containing zinc acetate (25 µmol). The residue was subjected to thin-layer chromatography (1000 µm), and the concentration of the Zn-N-ethylPP dimethyl ester was determined by UV-visible spectrophotometry as described above. The emission spectra of known amounts of N-ethylPP dimethyl ester were determined on a fluorescence plate reader. The solution (200 µl) was added to each well of a white 96-well microtiter plate and read on a fluorescence plate reader (Spectra MAX Gemini XS fluorescent plate reader/Softmax PRO software; Molecular Devices Corp., Sunnyvale, CA), using an excitation wavelength of 432 nm (Soret peak) and an emission scan from 600 to 800 nm. The relative fluorescence units at 630 nm were used to determine the points of a standard concentration curve. There was a linear relationship between the amounts of N-ethylPP dimethyl ester and relative fluorescence units (R² = 0.9841) (Fig. 3). The lower limit of detection is 2.5 pmol.

Preparation of Microsomes. Microsomes were isolated from male mice according to procedures used in our previous studies (Forkert, 1995). Livers from 3 male mice and lungs from 50 male mice were pooled for each microsomal sample. Human lung tissues were not pooled but were retained as individual samples. Human lung microsomes were prepared using procedures described previously (Forkert et al., 2001). Protein concentrations were determined using the method of Bradford (1976).

Microsomal Incubations. In murine microsomal incubations, reaction mix-
tures contained microsomal protein in 0.1 M phosphate buffer, pH 7.4, 2.0 mM NADPH, and 0 to 5.0 mM DASO₂ in a total volume of 1 ml. Protein concentrations used for murine and human liver microsomal incubations were 0.5 to 5 mg, and for murine and human lung microsomal incubations were 1 to 5 mg. Incubations with recombinant rat and human CYP2E1 were carried out with 50 to 200 pmol of enzyme, 2.0 mM NADPH, and 0 to 5.0 mM DASO₂. Incubations for time course experiments were carried out between 0 and 40 min. Incubations for concentration-response studies were performed with 1 to 4.0 mM DASO₂. All incubations were performed at 37°C. Controls included incubations performed in the absence of NADPH, DASO₂, or microsomes as well as incubations using boiled microsomes. After the incubations, N-alkylPP was isolated from the microsomes using the method described previously (Lavigne et al., 2002). The N-alkylPP was extracted into dichloromethane; the organic extract was washed with sodium bicarbonate (5% v/v) and water, reacted with zinc acetate (12 μmol) in methanol (1 ml), dried, and dissolved in methanol. For lung and liver samples, 200 μl of the suspension were added to each well of a white 96-well microtiter plate and read on a fluorescence plate reader (Spectra MAX Gemini XS fluorescence plate reader/Softmax PRO software), using an excitation wavelength of 432 nm (Soret peak) and an emission scan from 600 to 800 nm. The amounts of N-alkylPP present in the sample were determined by relating fluorescence values at 630 nm to the N-ethylPP dimethyl ester standard curve.

**Statistical Analysis.** Data in the in vivo studies are expressed as mean ± S.D. and were analyzed using Student’s t test. The level of significance was set at p < 0.05. Michaelis-Menten kinetic analysis was performed by using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

**Results and Discussions.**

Previous studies indicated that metabolism of DASO and DASO₂ favors oxidation at the terminal double bonds, yielding epoxides that conjugated with glutathione (Jin and Baillie, 1997). More recent studies corroborated these findings and showed that an epoxide (DASO₃), was formed in murine lung and liver microsomal incubations containing DASO₂ and NADPH, and was not formed in the absence of NADPH (Forkert et al., 2000; Premdas et al., 2000). The formation of DASO₃ coincided with loss of immunodetectable CYP2E1 protein and associated p-nitrophenol hydroxylation; these findings are in agreement with studies in rat liver suggesting that DASO₂ is metabolized by CYP2E1 (Brady et al., 1991a). The significant loss of heme associated with DASO₂ led us to undertake studies to test the hypothesis that the N-alkylPP adduct is generated from its metabolism.

Studies were carried out in mice treated with DASO₂ to determine whether the N-alkylPP adduct was formed in vivo. The identity of the N-alkylPP adduct was confirmed by characteristics of the absorption spectrum of the Zn-N-alkylprotoporphyrin IX as determined by UV-visible spectrophotometry; a major peak was observed at 432 nm and minor peaks at 547, 591, and 634 nm (Fig. 4A). The N-alkylPP

**Fig. 4.** Absorption spectrum (A) and formation (B) of the zinc-complexed N-alkylPP dimethyl ester in liver homogenates from female and male mice treated with DASO₂. *+, significantly different from levels in female mice.
adduct was detected in the livers of both male and female mice, with levels that were significantly higher in males than in females (Fig. 4B). The heme adduct was not detectable in the lungs of DASO2-treated mice. These results confirmed that the \( N \)-alkylPP adduct was formed in vivo from DASO2 in the liver but not in the lung. These findings are consistent with the considerably higher levels of cytochrome P450 found in the liver versus the lung, suggesting that the rates of adduct formation in the lung were too low to be detectable.

Identification of \( N \)-alkylPP in microsomal incubations was performed using a 96-well microtiter plate and a fluorescence plate reader. This strategy was adopted because lung microsomes from mice were of low yield, and the plate reader required only 200 \( \mu \)l of sample versus 2 ml in the cuvette for fluorometry as described in previous studies (Lavigne et al., 2002). Representative spectra obtained by fluorometry in liver and lung microsomal incubations are illustrated in Fig. 5. At an excitation wavelength of 432 nm, characteristic peaks were observed at 630 and 700 nm. The magnitudes of the peaks were greater in the liver (Fig. 5A) than in the lung (Fig. 5B) microsomal incubations. These peaks were not observed in microsomal incubations performed in the absence of NADPH (Fig. 5C) or DASO2 (Fig. 5D), nor were they found in incubations performed with boiled microsomes (data not shown).

In murine and human liver microsomal incubations, the rates of formation of \( N \)-alkylPP from DASO2 were both time-dependent and incremental from 0 to 30 min, with declines at 40 min (Figs. 6A and 7A). The rates of adduct formation by murine liver microsomes were incremental with protein concentrations ranging from 0.5 to 2.0 mg, with saturation rates at 2 to 3 mg of protein. In incubations of human liver microsomes, the rates of \( N \)-alkylPP formation increased with protein concentrations of 0 to 3 mg, with saturation rates of adduct formation at 3 to 5 mg (Fig. 7B). In murine lung microsomal incubations, rates of formation of the heme adduct were linear from 0 to 20 min and decreased thereafter (Fig. 8A). Rates of adduct formation were also protein-dependent and were linear from 0 to 4.0 mg of
N-ALKYLPROTOPORPHYRIN IX FORMATION FROM DIALLYL SULFONE

899

Protein, with saturation at 4 to 5 mg of protein concentration. Based on these data, DASO2 concentration studies in murine liver were carried out with 1.5 mg of microsomal protein for 10 min, in human liver with 1.5 mg of microsomal protein for 8 min, and in murine lung with 2.0 mg of microsomal protein for 10 min. The results showed that the rates of N-alkylPP adduct formation were highly correlated with DASO2 concentrations used in the incubations of microsomes from murine liver (R² = 0.9936) (Fig. 6C), human liver (R² = 0.9317) (Fig. 7C), and murine lung (R² = 0.9741) (Fig. 8C).

The results of Michaelis-Menten kinetic analysis of the DASO2 concentration-dependent studies are illustrated in Figs. 6C, 7C, and 8C, and the kinetic constants obtained are given in Table 1. The apparent Kₘ for murine liver microsomes was 2.9-fold lower than that for murine lung microsomes, which was similar to the apparent Kₘ for human liver microsomes. The apparent Vₘₐₓ for murine liver microsomes was similar to the apparent Vₘₐₓ for lung microsomes, but was about 2-fold lower than that in human liver microsomes. These data yielded apparent Vₘₐₓ/Kₘ ratios that were 1.3-fold higher for murine liver microsomes than for human liver microsomes. However, the Vₘₐₓ/Kₘ ratios for both murine and human liver microsomes were about 3- and 2-fold higher, respectively, than for murine lung microsomes. Formation of N-alkylPP was not detected in incubations of human lung microsomes. However, the values for the Vₘₐₓ and the Vₘₐₓ/Kₘ ratio found for the human liver microsomes suggested that formation of N-alkylPP may be a relevant event in DASO2 metabolism in the human.

Previous studies have reported that CYP2E1 is inhibited by DASO2 in lung and liver of mice (Forkert et al., 2000; Premdas et al., 2000). To determine the role of CYP2E1 in producing the N-alkylPP adduct, incubations of DASO2 were carried out with recombinant rat and human CYP2E1 enzymes. The heme adduct was generated in incubations of recombinant rat CYP2E1 with DASO2 in a time- and protein concentration-dependent manner (data not shown). Kinetic analysis of data from concentration-dependent studies (Fig. 9) yielded an apparent Kₘ of 0.35 ± 0.11 mM and an apparent Vₘₐₓ of 7.3 ± 0.9 pmol/min/nmol CYP2E1 (Vₘₐₓ/Kₘ = 20.86). These results supported the contention that the recombinant rat CYP2E1 enzyme has a high catalytic efficiency for formation of the N-alkylPP adduct, and is consistent with the inactivation of CYP2E1 found in previous studies (Premdas et al., 2000). Incubations of recombinant human CYP2E1 with DASO2 produced minimal rates of N-alkylPP formation and were too low for kinetic analysis.

In summary, the results of these studies demonstrated that metabolism of DASO2 leads to formation of the N-alkylPP adduct by murine liver and lung microsomes, by human liver microsomes, by recombinant rat CYP2E1, and in vivo in murine liver, and supported...

![Fig. 8. Time- (A) and protein-dependent (B) formation of the N-alkylPP adduct in incubations of murine lung microsomes with DASO2 (2.0 mM) and NADPH (2.0 mM). Kinetic analysis of incubations of the microsomes with various DASO2 concentrations and NADPH (C).](image)

![Fig. 9. Kinetic analysis of data from incubations of recombinant rat CYP2E1 (150 pmol) with DASO2 (2.0 mM) and NADPH (2.0 mM).](image)

![Table 1: Kinetic analysis of DASO2 metabolism to N-alkylPP in incubations of murine liver and lung microsomes and human liver microsomes](table)

<table>
<thead>
<tr>
<th>Reaction mixtures</th>
<th>Kₘ (mM)</th>
<th>Vₘₐₓ (nmol/min/mg protein)</th>
<th>Vₘₐₓ/Kₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine liver microsomes</td>
<td>0.85 ± 0.07</td>
<td>1.43 ± 0.03</td>
<td>1.68</td>
</tr>
<tr>
<td>Murine lung microsomes</td>
<td>2.48 ± 0.07</td>
<td>1.33 ± 0.11</td>
<td>0.54</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>2.41 ± 0.31</td>
<td>3.03 ± 0.19</td>
<td>1.26</td>
</tr>
<tr>
<td>Human lung microsomes</td>
<td>N.D.</td>
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<td></td>
</tr>
</tbody>
</table>

N.D., not detectable.
the contention that P450 inactivation by DASO₂ is associated with N-alkylation of the heme moiety.

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