Short Communication

FORMATION OF N-ALKYLPROTOPORPHYRIN 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 and in vitro was determined by spectrophotometric and fluorometric methods, respectively. 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 were 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 S-allylcysteine sulfoxide (alliin) by 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 (Sparnins et al., 1988).

Previous studies have identified both diallyl sulfoxide (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 (Brady 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 DASO as a CYP2E1 inhibitor has been ascribed to mechanism-based inactivation, and it is the metabolic event involving DASO 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 DASO against lung cytotoxicity induced by 1,1-dichloroethylene (Forkert et al., 1996a,b, 2000; Premdas et al., 2000). The results showed that DASO undergoes P450-dependent oxidation at one of its terminal double bonds to form diallyl sulfone monoepoxide (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 DASO (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 DASO (100 mg/kg p.o.). These findings suggested the possibility that bioactivation of DASO 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 DASO 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 DASO 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 sulfoxide; DASO₂, diallyl sulfone; DASO₃, 1,2-epoxypropyl-3,3′-sulfonyl-1′-propene.
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 (R<sub>f</sub> = 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<sup>-1</sup> cm<sup>-1</sup>) 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 (Kimmett 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 (R<sup>2</sup> = 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 \( \mu \)mol) in methanol (1 ml), dried, and dissolved in methanol. For lung and liver samples, 200 \( \mu \)l 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 fluorescent 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 \( \pi \)-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

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**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. *p* significantly different from levels in female mice.

**Fig. 5.** Fluorescence spectra obtained from incubation of DASO₂ (2.0 mM) and NADPH (2.0 mM) with liver (A) and lung (B) microsomes. Controls comprised incubations performed in the absence of DASO₂ (C) or NADPH (D). RFU, relative fluorescence units.
an 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 μl 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

![Fig. 6. Time- (A) and protein-dependent (B) formation of the N-alkylPP adduct in incubations of murine liver microsomes with DASO2 (2.0 mM) and NADPH (2.0 mM). Kinetic analysis of data from incubations of the microsomes with various DASO2 concentrations and NADPH (C).](image1)

![Fig. 7. Time- (A) and protein-dependent (B) formation of the N-alkylPP adduct in incubations of human liver microsomes with DASO2 (2.0 mM) and NADPH (2.0 mM). Kinetic analysis of data from incubations of the microsomes with various DASO2 concentrations and NADPH (C).](image2)
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/H₁₁₀₀₅₀.₉⁹₃₆) (Fig. 6C), human liver (R²/H₁₁₀₀₅₀.₉₃₁₇) (Fig. 7C), and murine lung (R²/H₁₁₀₀₅₀.₉₇₄₁) (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 mM and an apparent Vₘₐₓ of 7.3 pmol/min/mmol 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
the contention that P450 inactivation by DASO₂ is associated with N-alkylation of the heme moiety.

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