DIALLYL SULFONE, A DERIVATIVE OF GARLIC, INACTIVATES THE CYTOCHROME P450 ENZYME CYP2E1 BY FORMING *N*-ALKYLPROTOPORPHYRIN HEME ADDUCTS

by

DYLAN PAUL VINCENT BLACQUIERE

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ABSTRACT

Diallyl sulfone ($DASO_2$), a derivative of garlic formed by sequential oxidation of diallyl sulfide (DAS) and diallyl sulfoxide (DASO), has been shown to inhibit CYP2E1, a cytochrome P450 enzyme, in murine lung and liver. We tested the hypothesis that CYP2E1 inactivation occurs during $DASO_2$ oxidation through covalent binding of an alkyl group to the heme moiety of CYP2E1, resulting in the formation of an *N*-alkylprotoporphyrin adduct. Our objectives were to investigate the formation of these adducts in *in vitro* and *in vivo* systems to determine whether: i) these adducts are formed in murine lung and liver, ii) CYP2E1 is the target for heme alkylation, and iii) these adducts are also formed in human tissues. Liver and lung microsomes from mouse and human tissues were incubated with $DASO_2$, and examined for the formation of *N*-alkylprotoporphyrin adducts. Baculosomes (microsomes from baculovirus-infected insect cells transfected with rat CYP2E1 or CYP2B1 cDNA) were also incubated with $DASO_2$ and examined for adduct formation. In microsomal and baculosomal incubations, *N*-alkylprotoporphyrin adduct formation was protein-, time- and concentration-dependent. Baculosomes expressing CYP2E1 showed a twofold increase in formation of *N*-alkylprotoporphyrin adducts from $DASO_2$ when compared to baculosomes expressing CYP2B1. Male and female CD-1 mice were treated with $DASO_2$ (100 mg/kg) by oral gavage and investigated for the presence of *N*-alkylprotoporphyrins. Mice from all groups showed detectable levels of *N*-alkylprotoporphyrin adducts after administration of $DASO_2$. Pretreatment with acetone, which induces the CYP2E1 enzyme in the liver, resulted in a 41% increase in *N*-alkylprotoporphyrin adducts in liver microsomes from male mice. In *in vivo* studies, increases (30-40%) were also observed in male and female

mice. These findings indicated that CYP2E1 is an important target of heme alkylation by $DASO_2$, and that *N*-alkylprotoporphyrin heme adduct formation mediates CYP2E1 inactivation.

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"This is the great Theatre of Life. Admission is free, but the taxation is mortal. You come when you can, and leave when you must. The show is continuous. Good night."
-Robertson Davies

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LIST OF ABBREVIATIONS

4-ethyl DDC	=	3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihyropyridine
AIA	=	2-allyl-2-isopropylacetamide
C	=	Celsius
CYP2B1	=	cytochrome P450 2B1
CYP2C11	=	cytochrome P450 2C11
CYP2E1	=	cytochrome P450 2E1
d	=	days
DAS	=	diallyl sulfide
DASO	=	diallyl sulfoxide
$DASO_2$	=	diallyl sulfone
$DASO_3$	=	1,2-epoxypropyl-3,3'-sulfonyl-1'-propene
DCE	=	dichloroethylene
DCM	=	dichloromethane
DDC	=	3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine
EDTA	=	disodium ethelyenediamine tetraacetate
h	=	hours
min	=	minutes
mM	=	millimolar
mRNA	=	messenger ribonucleic acid
NADPH	=	nicotinamide adenine dinucleotide phosphate
NDMA	=	*N*-nitrosodimethylamine
nmol	=	nanomoles
PB	=	phenobarbital

pmol	=	picomoles
TCE	=	trichloroethylene
TLC	=	thin-layer chromatography
μmol	=	micromoles
UV	=	ultraviolet

CHAPTER ONE

INTRODUCTION

Cytochrome P450

The cytochrome P450 family of proteins has been studied for many years in human pharmacology and toxicology, and the role of these proteins in the biotransformation of xenobiotic substances is well documented (Correia 2001, Nelson et al. 1996). These reactions result in the conversion of harmful substances to forms that can be excreted from the body with relative ease when compared to the parent forms. Biotransformation by cytochrome P450 and modification of xenobiotics is referred to as phase I biotransformation by toxicologists (Correia 2001). There are many enzymes in the cytochrome P450 superfamily (White and Coon 1980), each localized to specific species and tissues, though overlap occurs to various extents.

Cytochrome P450 enzymes consist of a prosthetic heme group bound to a protein that spans the membranes of various organelles, such as the smooth endoplasmic reticulum, in which the enzyme is found (Omura and Sato, 1964, Nelson et al. 1996). The heme group contains a molecule of iron, which accepts electrons from cytochrome P450 reductase and then catalyzes the oxidative reactions that metabolize the enzyme's substrates to alternate forms (Nelson et al. 1986). Each enzyme reacts with specific substrates to form metabolic by-products, which are further modified to form readily excretable by-products. For example, CYP2E1 is crucial in metabolizing and detoxifying *N*-nitrosodimethylamine (NDMA) (Yang et al. 1990) as well as other toxic substances including ethanol (Koop et al. 1982) and chloroxazone (Peter et al. 1990).

Different tissues contain varying amounts of cytochrome P450 enzymes. The liver, which is the major site of xenobiotic biotransformation, contains a higher concentration of cytochrome P450 enzymes than the kidney or the lung (Correia 2001). Several studies have examined gender and age differences in the amounts of particular cytochrome P450 enzymes. In various strains of mice, the levels of certain cytochrome P450 enzymes in the kidney are several-fold higher in males than in females (Hawke and Welch 1985). In contrast, the levels of CYP2E1 in the lung are slightly higher in females than in males (Forkert et al. 1996a), while no significant difference was found between sexes in CYP2E1 content of the liver (Hong et al. 1987). However, the difference in cytochrome P450 amounts may not be reflected in the actual capacities for bioactivation of xenobiotics modified by CYP2E1 (Forkert et al. 1996b).

Different enzymes in the P450 family are involved in the biotransformation of various chemicals. Some substrates can be modified by several P450 enzymes, though the reactions are usually specific to certain P450 types. As well, the activities of P450 enzymes can be induced by various substrates, including phenobarbital (Wong and Marks 1999), ethanol (Kurata et al. 1991) and acetone (Forkert et al. 1994). Treatment with these substances increases the efficacy of the biotransformation system, allowing more substrate to be converted by cytochrome P450 into more readily excretable metabolites. The extent of cytochrome P450 induction depends on the length of exposure and the inducer being used (Forkert et al. 1994). Hepatic CYP2E1 can be cumulatively induced after long-term exposure to ethanol in mice and humans, but pretreatment with acetone showed only short-term induction that did not accumulate over time (Forkert et al. 1994). However, treatment with acetone produced elevated levels of CYP2E1 after pretreatment, as did phenobarbital on levels of hepatic CYP2B1. Conversely, some substances have

been found to inhibit the activity of P450 enzymes, resulting in a reduced ability to modify xenobiotic substrates. One of these substances is a compound found in the garlic plant, diallyl sulfide (DAS), as well as its CYP2E1-mediated oxidation products diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) (Yang et al. 1994, Forkert et al. 1996a). Other inhibitors include 2-allyl-2-isopropylacetamide (AIA), which inhibits CYP2C11 and CYP2B1 (Ortiz de Montellano and Mico 1981, Wong and Marks 1999) and diethyldithiocarbamate, which is also selective for CYP2E1 (Guengerich et al. 1991).

Cytochrome P450-Mediated Bioactivation

In many cases, the changes caused by P450 enzymes result in the formation of a less toxic substance that is more readily excretable by the body. However, certain chemicals are converted to toxic species when metabolized by cytochrome P450 enzymes. One example is 1,1-dichloroethylene (DCE), an intermediate compound used in the production of plastics, and trichloroethylene (TCE), a related compound. DCE, when administered intraperitoneally to mice, was shown to produce damage to the Clara cells in lung bronchioles (Forkert et al. 1986, 1996a). DCE was also shown to produce a wide array of toxic effects in the liver (Reynolds et al. 1975). Further studies showed that the damaging agents were metabolites of DCE formed from the oxidation of DCE, catalyzed by CYP2E1. A similar mechanism was observed for TCE (Forkert et al. 2002). CYP2E1-mediated damage from these compounds was observed in a wide range of tissues, including lung, liver, epididymis and testis (Lee and Forkert 1995, Forkert 1999, Forkert et al. 2002). In these cases, the CYP2E1-mediated biotransformation of these compounds forms toxic metabolites that cause necrotic damage to the target tissues, resulting in numerous symptoms of DCE-related toxicity (Lee and Forkert 1995). Other chemicals,

such as vinyl carbamate, have been shown to exert their toxic effects after bioactivation by P450 enzymes (Guengerich et al. 1991, Titus and Forkert 2001). This results in a paradoxical effect – the biological system that prevents the toxic effects of one substance may inadvertently exacerbate the toxic effects of another. To that end, research aimed at reducing the effects of these compounds has focused on the interactions of these xenobiotics with cytochrome P450, and potential mechanisms for disrupting these reactions and preventing the formation of harmful metabolites have been examined (Yang et al. 1994).

Inactivation of Cytochrome P450

Many reactions catalyzed by cytochrome P450 result in the generation of electrophilic metabolites, which react with other molecules. In some cases, these metabolites may attack cytochrome P450 itself, resulting in its inactivation (Ortiz de Montellano et al. 1978). Certain chemicals, such as AIA, may act as such selective inhibitors and may prevent the toxic and carcinogenic effects of various xenobiotics and their metabolites (Ortiz de Montellano and Mico 1981, Wong and Marks 1999, Yang et al. 1994).

There are three generally recognized mechanisms for the irreversible inactivation of P450 enzymes by exogenous compounds – the covalent binding of the electrophilic metabolite to the apoprotein of the cytochrome (Halpert and Neal, 1981), the binding of the metabolite to the prosthetic heme group (Ortiz de Montellano and Correia 1995), and the activation of the heme group to form a product that binds irreversibly to the microsomal protein (Davies et al. 1986). All of these mechanisms prevent the enzyme from reacting with other substrates, reducing the ability of the cytochrome to

biotransform xenobiotic compounds (Guengerich 1986, Wong et al. 1999). Some of these mechanisms may occur in tandem, resulting in a varied mechanism for cytochrome P450 inactivation (Davies et al. 1986). Previous studies have noted that compounds containing terminal double bonds, or allyl groups, can cause the loss of hepatic cytochrome P450, as detected by the formation of green pigments with thin-layer chromatography (De Matteis and Cantoni 1979). Previous investigations have noted that after treatment with these compounds, spectrometric studies have observed the conversion of the heme moiety of the cytochrome P450 enzyme to a modified porphyrin form, with components of the xenobiotic bound to one of the four pyrrole rings of the prosthetic heme group (Marks 1986). The mechanism for this formation, observed with AIA, involves the cytochrome P450-mediated metabolism of the allyl-containing xenobiotic to a derivative form, which in turn binds to the heme group and inactivates the enzyme (Guengerich 1986, Ortiz de Montellano and Mico 1981). Some studies have suggested that the modified heme group may dissociate from the apoprotein, and postulate that the heme may be replenished from exogenous heme molecules, allowing for the regeneration of functional cytochrome (Ortiz de Montellano and Mico 1981, Wong and Marks 1999). However, this auxiliary supply of heme would eventually be depleted, leading to the functional inhibition of cytochrome P450 (Marks 1986, Wong and Marks 1999). In addition, some of the alkylated heme adducts may act as inhibitors of enzymes in the heme biosynthetic pathways, resulting in the accumulation of heme precursors and the development of experimental porphyria (De Matteis and Marks 1996). Further degradation of the heme adduct may result in the binding of heme fragments to the apoprotein, resulting in the destruction of the enzyme (Davies et al. 1986).

N-alkylprotoporphyrin Adducts

During reactions with chemicals such as AIA (Ortiz de Montellano and Mico 1981) and *N*-ethyl DDC (Riddick et al 1989), the heme group of cytochrome P450 is inactivated and transformed into a green pigment, which fluoresces red under long-wave ultraviolet light (Marks 1986). Compounds that produce this pigment are referred to as porphyrinogenic, as they interfere with the integrity of the porphyrinogenic pathway that creates functional heme molecules (Marks 1986, De Matteis and Marks 1996). These compounds share numerous structural similarities, including multiple bonding in the form of olefins, acetylene groups, or allyl groups (Marks 1986). The porphyrinogenic effect is most pronounced after treatment with chemicals containing terminal allyl groups (De Matteis and Cantoni 1979). Some compounds, such as 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihyropyridine (4-ethyl DDC) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), interfere with the activity of enzymes in the biosynthetic pathway that creates heme, such as ferrochelatase (Correia et al. 1987, Marks 1986). Others, such as AIA, react directly with the heme molecule after its incorporation into the cytochrome P450 enzyme (Ortiz de Montellano et al. 1978). The observed mechanism involves the covalent alkylation of the heme molecule at one of the four pyrrole rings (Figure 1), coupled with the loss of the iron molecule that allows oxidation reactions to occur at the enzyme's active site (Wong and Marks 1999). This results in the formation of an *N*-alkylprotoporphyrin IX molecule which may inhibit the activity of other molecules in the heme metabolic pathway and cause the accumulation of protoporphyrin IX (Wong and Marks 1999). Accumulation of the *N*-alkylprotoporphyrin adducts is observed as a green pigment that can be detected using thin-layer chromatography and UV-visible light spectrophotometry (Ortiz de Montellano and Mico 1981). The formation of these

compounds appears to be a mechanistic process that depends upon NADPH and oxygen (Ortiz de Montellano and Mico 1981). As with the activity of cytochrome P450, the amount of these adducts can be increased or decreased by pretreatment with inducers (such as phenobarbital or acetone) or inhibitors of P450 enzymes, respectively (Ortiz de Montellano et al. 1978).

Diallyl Sulfone

Diallyl sulfone ($DASO_2$) is a metabolite of diallyl sulfide (DAS), a compound which is found in the garlic plant (*Allivum sativum*) (Forkert et al. 2000, Yang et al. 1994) (Figure 2). DAS can be formed as a metabolic product of other compounds found in garlic, in the processes of cooking, and after ingestion (Hayes et al. 1987). The anticarcinogenic effects of these compounds have been examined in previous studies, where DAS has been linked to the prevention of carcinogenesis caused by chemicals such as benzo[α]pyrene (Sparnins et al. 1988), aflatoxin B_1 (Haber-Mignard et al. 1996) and dimethylhydrazine (Wargovitch 1987). Diallyl sulfoxide (DASO) and $DASO_2$ have been detected in extracts of liver and in blood and urine samples from rats treated with DAS (Brady et al. 1991). $DASO_2$ has been shown to be formed by progressive oxidation reactions of DAS (Jin and Baillie 1997) (Figure 3). Microsomal studies have further suggested that the transformation of DAS to $DASO_2$ is dependent upon NADPH, and that CYP2E1 appears to be the enzyme involved in DAS metabolism (Brady et al. 1991; Premdas et al. 2000). Treatment of rat liver microsomes with DAS has shown a time- and concentration-dependent decrease in the microsomal content and catalytic activity of CYP2E1 (Brady et al. 1988). However, treatment of rats with DASO and $DASO_2$ resulted in larger decreases in the amount and activity of CYP2E1, with the largest decrease

occurring after treatment with $DASO_2$ (Brady et al.1991), a compound that contains readily reactive allyl groups.

The metabolite $DASO_2$ appears to be the most active of the metabolites of DAS. It has been shown to act as a specific inhibitor of CYP2E1 in murine lung and liver (Forkert et al. 1995; Premdas et al. 2000, Brady et al. 1991). $DASO_2$ also inhibits the activity of CYP2E1-mediated *p*-nitrophenol hydroxylation, an enzymatic activity that is a catalytic marker for CYP2E1 (Forkert et al. 1995). As well, when treated with $DASO_2$, the damage done to Clara cells in murine lung after treatment with DCE was decreased. There were also reductions in the levels of 2-(*S*-glutathionyl)-acetyl glutathione and of 2-*S*-glutathionyl acetate, the glutathione conjugates of DCE that are formed through oxidative reactions catalyzed by CYP2E1 (Forkert 1999). Taken together, these results show that $DASO_2$ may prevent the toxic effects observed in the lung following treatment with DCE. However, the precise mechanism for these effects remains unclear.

It is believed that the activity of $DASO_2$ upon CYP2E1 is based upon a mechanistic process that involves the reaction of the terminal allyl groups of $DASO_2$ with the enzyme, forming a reactive epoxide (Premdas et al. 2000). Forkert et al. (2000) hypothesized that the epoxide $DASO_3$ was responsible for alkylating CYP2E1 and rendering it unavailable for further metabolic activity. Studies with *p*-nitrophenol hydroxylase assays, which are used to determine the catalytic activity of CYP2E1, showed an inverse relationship between the catalytic activity of CYP2E1 in murine lung microsomes and the presence of the $DASO_3$ epoxide (Forkert et al. 2000). However, studies with other chemicals that are known to form alkylated heme adducts suggest that epoxides formed through metabolism of porphyrinogenic chemicals may not be directly responsible for the alkylation of the heme moiety. The possibility has been raised that the

heme group is alkylated by a cationic intermediate formed as the chemical is metabolized by cytochrome P450 (Ortiz de Montellano et al. 1979). However, the precise mechanism for the inactivation of CYP2E1 by $DASO_2$ remains unclear.

Hypothesis and Objectives

In this study, we will test the hypothesis that the metabolism of $DASO_2$ by CYP2E1 results in the formation of *N*-alkylprotoporphyrin adducts. To test this hypothesis, the following objectives will be addressed:

1) To determine that *N*-alkylprotoporphyrin adducts are formed in lung and liver after the administration of $DASO_2$ to mice.
2) To determine that CYP2E1 is involved in the formation of *N*-alkylprotoporphyrin adducts after exposure to $DASO_2$.
3) To determine that *N*-alkylprotoporphyrin adducts are observed in human lung and liver after exposure to $DASO_2$.

CHAPTER TWO

MATERIALS AND METHODS

Materials

Chemicals and reagents were provided by standard suppliers as follows: Fisher Scientific Co. (Nepean, Ontario): sodium bicarbonate, potassium chloride, disodiumethylenediamine tetraacetate (EDTA), methanol (high-pressure liquid chromatography grade) and acetone (high-pressure liquid chromatography grade); Sigma Chemical Co (St. Louis, Missouri): zinc acetate, potassium phosphate, dichloromethane (> 99% purity) and anhydrous sodium sulfate. Aldrich Chemical Co (Toronto, Ontario): iodoethane; Frontier Scientific Porphyrin Products (Logan, Utah): protoporphyrin IX dimethyl ester; VWR International (Mississauga, Ontario): 96-well microtitre plates for fluorometric scanning. Diallyl sulfone ($DASO_2$) was obtained from Colour Your Enzyme, Bath, Ontario. 2-allyl-2-isopropylacetamide (AIA) was generously donated by Dr. Gerald Marks, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario.

Treatment of Animals

CD-1 mice (male and female) of 25-28 g body weight were obtained from Charles River Canada (St. Constant, Quebec, Canada). The animals were housed in the Animal Care Facility of Queen's University and maintained on a 12 h light/dark cycle. The mice were given free access to food and water and acclimated for at least seven days prior to initiation of experiments. In studies involving the induction of CYP2E1, mice were treated with 1% acetone in drinking water for eight days as described in Forkert et al.

(1994). Mice were separated into groups of ten, and treated with 100 mg/kg AIA by i.p. injection, or with 100 mg/kg $DASO_2$ by oral gavage. In both cases, distilled water was used as a negative control. After 4 h (AIA) or 2 h ($DASO_2$), mice were anaesthetized with sodium pentobarbital (0.12 mg/g mouse). The chest wall was opened, and the mice were perfused with ice cold 1.15% KCl, administered through the left ventricle of the heart, with outflow eliminated through the right atrium. Once tissues were drained of blood, lungs and livers were excised, blotted dry, and weighed. Tissues were then minced with scissors and homogenized in ice-cold 5% H_2SO_4/methanol (approximately 30 mL per 3 livers/10 lungs). Tissues were then homogenized using an Erberbach tissue homogenizer, and stored overnight in a coldroom maintained at 2° C.

Spectrophotometric/Fluorometric Methods

When complexed with zinc and viewed in a UV-visible light spectrophotometer, *N*-alkylprotoporphyrin adducts exhibit a characteristic absorption spectrum with a large peak at approximately 432 nm, known as the Soret peak (Wong and Marks 1999). The absorbance at this peak was used to quantify the amount of *N*-alkylprotoporphyrin adducts by dividing the absorbance by the molar extinction coefficient of zinc-complexed *N*-alkylprotoporphyrin adducts. The coefficient of *N*-ethylprotoporphyrin, approximated as 128, 000 $m^{-1}cm^{-1}$ at 432 nm, was used to approximate those of the unknown alkylated heme adducts (Wong and Marks 1999). Using this method, auxiliary peaks are observed at 547 nm, 591 nm and 634 nm, respectively (Figure 4) though the wavelengths of these peaks may vary depending upon the individual structure of the compound (Ortiz de Montellano et al. 1978). These spectra do not appear if the adducts are not formed.

When observed using fluorometry with an excitation wavelength of 432 nm, a distinctive pattern of emission spectra is observed, with peaks observed at 640 nm and 700 nm (Figure 5), allowing for the detection and quantification of these heme compounds. These wavelengths differ from those reported by Lavigne et al. (2002) who saw emission peaks at 660 and 720 nm. This difference can be attributed to the different chemical structures of the *N*-alkylprotoporphyrin adducts (Lavigne et al. 2002).

Synthesis of Zn *N*-ethylprotoporphyrin standards

In order to quantify the amount of heme adducts observed after *in vitro* incubations with microsomal proteins, a standard curve was prepared using *N*-ethylprotoporphyrin as an analog for the *N*-alkylprotoporphyrin adducts formed after treatment with $DASO_2$. This compound has been used in previous studies as a comparable standard to other alkylated heme adducts in fluorometric studies (Lavigne et al. 2002).

To synthesize the N-ethylprotoporphyrin, protoporphyrin-IX dimethyl ester (3.2 mg) was reacted with iodoethane (2 mL) and left at 105° C for 18 h in a closed 5 mL test tube. The reaction mixture was cooled to room temperature and the remaining iodoethane was evaporated under nitrogen. The residue was dissolved in 2 mL dichloromethane (DCM) and applied to a 2000 μm thin-layer chromatography plate (silica, 20 cm x 20 cm). The plate was developed in a 20:3 (v/v) solution of DCM and methanol for 1 h, and then examined for red fluorescent bands (corresponding to reacted dimethyl esters) under long-wave ultraviolet light. The silica gel containing the band was scraped from the plate and then mixed with 20 mL of acetone in 50 mL round-bottomed polypropylene copolymer centrifuge tubes. The mixtures were centrifuged at 200g for 5 min at room temperature, after which the supernatant was removed, and the acetone-extraction and

centrifugation steps were repeated until the entire product had been collected. Acetone extracts were pooled and then evaporated to dryness. The residue was resuspended in 2 mL methanol containing 25 μmol zinc acetate, and re-evaporated. This residue was dissolved in 2 mL DCM and applied to a 1000 μm thin-layer chromatography plate (silica, 20 cm x 20 cm). The plate was developed in a 5:1 (v/v) solution of DCM and acetone for 1 h, and then examined for red fluorescent bands under long-wave ultraviolet light. The bands were then scraped off and extracted in acetone as above. The final residue was then suspended in 2 mL DCM and examined using ultraviolet-visible light spectrophotometry to determine the concentration of zinc-complexed *N*-ethylprotoporphyrin IX molecules.

Preparation of *N*-ethylprotoporphyrin standard curve

Known amounts of *N*-ethylprotoporphyrin IX were placed in 200 μL wells of a 96-well microtitre plate. The plate was then read on a fluorescence plate reader using an excitation wavelength of 432 nm. The emission spectra were recorded, and the relative fluorescence units at 630 nm were used to determine the points of a standard concentration curve (Figure 6). To facilitate the observation of *N*-alkylprotoporphyrin adducts, a fluorescence plate reader was used.

Preparation of Microsomes

Male CD-1 mice were placed in groups of ten and sacrificed by cervical dislocation, after which tissues (lung, liver) were obtained, minced with scissors and homogenized in four volumes of ice-cold phosphate buffered KCl (1.15% KCl, 10 mM K_2HPO_4, pH 7.4). The homogenate was centrifuged at 9000g for 20 min at 4° C, and the

supernatant was collected. The supernatant was centrifuged at 106,000g for 60 min at 4° C, and the resultant pellet was homogenized in ice-cold phosphate buffered KCl. The homogenate was then centrifuged at 106,000g for 60 min at 4° C. The final pellet was resuspended in ~2 mL of 0.1 M K_2HPO_4 buffer (pH 7.4) containing 1.5 mM EDTA. The microsomal suspension was then divided into aliquots of 10 – 500 μL and stored at -80° C. A sample of microsomal suspension was set aside for determination of protein concentration using the Bradford method of protein determination (Bradford 1976).

Samples of human lung were obtained from Kingston General Hospital, using tissue removed from patients undergoing lobectomy procedures. The tissue was minced with razors and homogenized using a Polytron tissue homogenizer. The homogenate was then centrifuged and lung microsomes prepared using methods described above. All tissue was prepared using Biohazard Level-2 precautions as outlined by the Queen's University Department of Environmental Health and Safety. For human liver microsomal experiments, microsomes were obtained from Gentest (Woburn, Massachusetts) were aliquoted and placed in incubations as above, using Biohazard Level-2 precautions.

Microsomal Incubations

Incubations were performed in a total volume of 1 mL. Depending upon the experimental protocol, 1-5 mg of protein (mouse) or 150 pmol of cytochrome P450 (human) were incubated with 1-5 mM $DASO_2$ (100 mM stock) and 2.0 mM NADPH (100 mM stock). The incubation was brought to a total volume of 1 mL with the addition of 0.1 M K_2HPO_4 buffer (pH 7.4). The solutions were mixed and then incubated at 37° C for 30-120 minutes, depending on the experimental protocol. Incubations performed without NADPH and/or without $DASO_2$ were used as negative controls. After the

determined time had elapsed, incubations were placed in 4 mL of ice-cold 5% H_2SO_4/methanol and left overnight at 2° C.

Baculosome Incubations

Rat CYP2E1 + P450 reductase and CYP2B1 + P450 reductase microsomes (Gentest, Woburn, Massachusetts) were thawed and used in microsomal incubations as described above. Incubations were performed in a total reaction volume of 1 mL using the same methods as described above. Incubations performed without NADPH and/or without $DASO_2$ were used as negative controls. CYP2E1 incubations were performed using 150 pmol of cytochrome P450 and 0.5–3 mM $DASO_2$ for an incubation time of 60 min at 37° C. In the cytochrome P450 enzyme comparison experiments, incubations were performed using 150 pmol of CYP2E1 or CYP2B1 and 3 mM $DASO_2$ for 60 min at 37° C. Following the incubations, the samples were placed in 4 mL of ice-cold 5% H_2SO_4/methanol and left overnight at 2° C.

Dichloromethane Extraction

After at least 12 h in the dark at 2° C, the microsomal reaction mixtures were added to 5 mL distilled water, and extracted with 15 mL DCM in a separatory funnel. The resultant solution was then rinsed with 10 mL dichloromethane (DCM) and 15 mL distilled water. After the final separation, 1 mL of zinc acetate (25 μmol) was added and the extract was evaporated to dryness. Samples were stored at –20° C until analysis, then redissolved in 2 mL methanol. The suspensions were placed in 200 μL wells on a 96-well microtitre plate and read on a fluorescence plate reader with an excitation wavelength of

432 nm. The emission spectra were recorded and fluorescence levels analyzed using the prepared standard curve.

Porphyrin Extraction

The tissue homogenate was vacuum-filtered using #2 Whatman paper. The filtrate was then mixed with an equivalent volume of water, and then extracted twice with 60 mL of DCM in a separatory funnel. The extract was then rinsed once with 80 mL of sodium bicarbonate (5%) and twice with 80 mL of distilled water. The remaining water was removed by adding anhydrous sodium sulfate for 60 min, followed by filtration. The final extract was then placed in a 100 mL Rotovap flask and dried using a rotary evaporator. When ~20 mL of extract remained, 2 mL of zinc acetate (25 μmol) was added and the extract was evaporated to dryness.

Thin-Layer Chromatography

The dried extract residue was dissolved in 2 mL DCM and applied to a 2000 μm thin-layer chromatography plate (silica, 20 cm x 20 cm). The plate was developed in a 260:39 (v/v) solution of DCM and methanol for 1 h, and observed for red fluorescent bands under long-wave ultraviolet light. The band was removed from the plate by scraping, and then mixed with 20 mL of acetone in 50 mL round-bottomed polypropylene copolymer centrifuge tubes. The mixtures were centrifuged at 1750 g for 5 min at room temperature. The supernatant was removed, and the acetone-extraction and centrifugation steps were repeated until the supernatant no longer appeared pink under long-wave ultraviolet light. The acetone extracts were pooled and then evaporated to dryness. The residue was resuspended in 2 mL methanol containing 25 μmol zinc acetate, and re-

evaporated. This residue was dissolved in 2 mL DCM and examined using ultraviolet-visible light spectrophotometry. If the sample was not of sufficient purity to clearly observe the absorbance pattern of N-alkylprotoporphyrin adducts, the sample was evaporated to dryness, resuspended in 2 mL DCM and applied to a 1000 μm thin-layer chromatography plate (silica, 20 cm x 20 cm). The plate was developed in a 5:1 (v/v) solution of DCM and acetone for 1 h, and then examined for red fluorescent bands under long-wave ultraviolet light. The bands were then scraped from the plate and extracted in acetone as above. The final residue was then suspended in 2 mL DCM and examined using UV-visible light spectrophotometry to determine the concentration of zinc-complexed *N*-alkylprotoporphyrin IX.

Statistical Analysis

For standard curve determination, linear regression analysis was used to determine the relationship between fluorescence and *N*-ethylprotoporphyrin adduct concentrations. Comparisons between CYP2E1 and CYP2B1 baculosome incubations were analyzed with the Student's t-test. Comparisons of microsomal incubations between control and acetone-treated mice were made using the Mann-Whitney Rank Sum Test. The *in vivo* comparisons between gender groups and between acetone-treated and control mice were analyzed using one-way ANOVA followed by the Tukey Test. Data are presented as mean ± standard deviation. Significance was set at $p < 0.05$.

CHAPTER THREE

RESULTS

Hepatic Microsomal Incubations

To determine the levels of *N*-alkylprotoporphyrins formed in microsomal incubations, the evaporated residues of the microsomal extracts were analyzed using a fluorescence plate reader, a method more sensitive than UV-visible light spectrophotometry (Lavigne et al. 2002). All experiments consisted of four microsomal incubations from two separate microsome preparations; each preparation conatined the pooled liver tissue of ten mice ($n = 4$). To determine whether the formation of these adducts formation was protein-dependent, liver microsomal incubations were performed using varying concentrations of hepatic microsomal protein, ranging from 0.5 mg/mL to 3.0 mg/mL. The microsomes were incubated with a concentration of 2 mM of $DASO_2$. The amount of *N*-alkylprotoporphyrin adducts formed in the incubations increased with elevated concentrations of protein (Figure 7). At 3 mg/mL, 1.2 nmol of *N*-alkylprotoporphyrins were detected, an 800% increase from the amount seen at 0.5 mg/mL.

To determine the effects of increased concentrations of $DASO_2$ on the formation of alkylated heme adducts in liver, microsomal incubations were prepared using a protein concentration of 3 mg/mL. Varying concentrations of $DASO_2$ ranging from 1 to 5 mM were added to the incubations. The amount of heme adducts formed increased with increasing concentrations of $DASO_2$, with the highest amounts (0.92 nmol) detected at a concentration of 4 mM (Figure 8). At higher concentrations, there was a slight decrease in the amount of *N*-alkylprotoporphyrins observed.

In order to determine the effect of incubation time upon the quantity of alkylated heme adducts found, incubations were performed using 3 mg/mL of hepatic microsomal protein and 4 mM $DASO_2$. The mixtures were incubated for lengths of time ranging from 30 min to 120 min, and then extracted and quantified using the fluorometric methods described above. The amount of *N*-alkylprotoporphyrins was highest (1.13 nmol) at 60 min, after which the amounts decreased to a low of 0.98 nmol at 120 min (Figure 9).

To examine the effect of a CYP2E1 inducer upon the levels of *N*-alkylprotoporphyrin adducts formed *in vitro*, microsomes were prepared from male CD-1 mice pretreated with 1% acetone for 8 d. Previous studies have shown that chronic treatment with acetone produces a 5.3-fold induction of CYP2E1 (Forkert et al. 1994). The microsomes were then incubated with 4 mM $DASO_2$, as were microsomes from untreated mice. Following treatment, the microsomes were isolated, incubated, extracted and quantified as described above. Microsomes from tissues pretreated with acetone showed a significant 41% elevation in the amount of N-alkylprotoporphyrin adducts formed when compared to that in microsomes from untreated mice. (Figure 10).

Human Microsomal Incubations

To determine if *N*-alkylprotoporphyrin adducts were formed in human tissue after exposure to $DASO_2$, incubations were performed using microsomes prepared from human lung and liver. Microsomal proteins containing 150 pmol of cytochrome P450 was added to the incubation, along with varying concentrations of $DASO_2$ (1-5 mM). Incubation mixtures were then extracted and heme adducts quantified as outlined above. All experiments consisted of four microsomal incubations prepared from human tissue sources ($n = 4$). A concentration-dependent response was observed in human liver

incubations with a maximum of 0.049 nmol of *N*-alkylprotoporphyrin observed when incubated with 4 mM of $DASO_2$, and a slight decrease at higher concentrations (Figure 11). Trace amounts of *N*-alkylprotoporphyrin adducts were detected in incubations of human lung microsomes and were not quantifiable (data not shown).

Pulmonary Microsomal Incubations

The perfused lungs of male CD-1 mice were pooled and microsomes were prepared from the tissue. All experiments consisted of four microsomal incubations from four separate microsomal preparations; each preparation contained the pooled lung tissue of thirty mice ($n = 4$). Preliminary experiments showed that incubations of lung microsomes with $DASO_2$ resulted in the formation of *N*-alkylprotoporphyrin adducts that could be quantified by fluorometric analysis.

To determine whether the protein content affected the formation of *N*-alkylprotoporphyrin adducts, varying concentrations (1-5 mg/mL) of lung microsomal protein were incubated with 4 mM $DASO_2$ using the methods described above. The amount of heme adducts increased with increasing levels of microsomal protein to a maximum of 0.270 nmol at 4 mg/ml of microsomal protein (Figure 12). At 5 mg/mL, the amount of *N*-alkylprotoporphyrins decreased slightly, but levels were still nearly tenfold higher than levels observed at 1 mg/mL of protein.

To examine the effects of increased concentration of $DASO_2$ upon the amount of heme adducts formed, microsomal incubations were carried out with 4 mg/mL of lung microsomal protein, along with concentrations ranging from 1-5 mM $DASO_2$. The quantity of *N*-alkylprotoporphyrins observed after incubation with 2 mM $DASO_2$

increased almost fourfold from levels observed at 1 mM; levels then plateaued to show a steady amount of adducts at higher concentrations (Figure 13).

To determine the effects of incubation time, incubations were prepared using 5 mg/mL of pulmonary microsomal protein and 4 mM $DASO_2$ and were incubated for 30-120 min. The highest quantities of adducts (0.21 nmol) were observed at 60 min, with a subsequent 50% decrease at 90 min (Figure 14).

Baculosome Incubations

To examine the formation of *N*-alkylprotoporphyrin adducts in a closed system with a single cytochrome P450 enzyme for reaction, incubations were performed using baculosomes expressing rat CYP2E1 or CYP2B1. Incubations were prepared with microsomal protein containing 150 pmol of cytochrome P450, and 0.5 - 3 mM $DASO_2$, The incubations were allowed to react for 60 min, after which the residues were extracted, and examined for heme adducts as outlined above. A concentration response was observed, showing an increase in the amount of *N*-alkylprotoporphyrins from 0.022 nmol at 0.5 mM of $DASO_2$ to 0.045 nmol at 2 mM $DASO_2$, though a subsequent decrease in the amount of heme adducts was observed at 3 mM (Figure 15).

To determine whether CYP2E1 was a specific target of $DASO_2$, incubations were performed with baculosomes containing 150 pmol of rat CYP2E1-expressed or rat CYP2B1-expressed microsomes and 3 mM $DASO_2$. *N*-alkylprotoporphyrin adducts were observed in both CYP2E1 and CYP2B1 incubations, though twice as many adducts were formed in the CYP2E1 baculosome incubations than in the CYP2B1 preparations (Figure 16).

In Vivo Treatments

To determine the efficacy of the *in vivo* model, AIA-mediated formation of *N*-alkylprotoporphyrins was examined *in vivo*. *N*-alkylprotoporphyrins were formed in detectable amounts (data not shown). The amount of *N*-alkylprotoporphyrin adducts formed after treatment with $DASO_2$ were then determined in both male and female mice divided into control and acetone-treated groups (Figure 17). Female mice treated with acetone showed a 41% increase in the amount of *N*-alkylprotoporphyrin adducts when compared to untreated female mice; however, this difference was not statistically significant ($p > 0.05$). There was a significant difference in the amount of alkylated heme adducts formed in untreated male mice, which was 50% higher than the quantity of adducts formed in untreated female mice ($p < 0.05$). As well, there was a significant increase of 30-40% in *N*-alkylprotoporphyrin adducts in male mice treated with acetone when compared to male mice in the control group, and to female mice treated with acetone ($p < 0.05$).

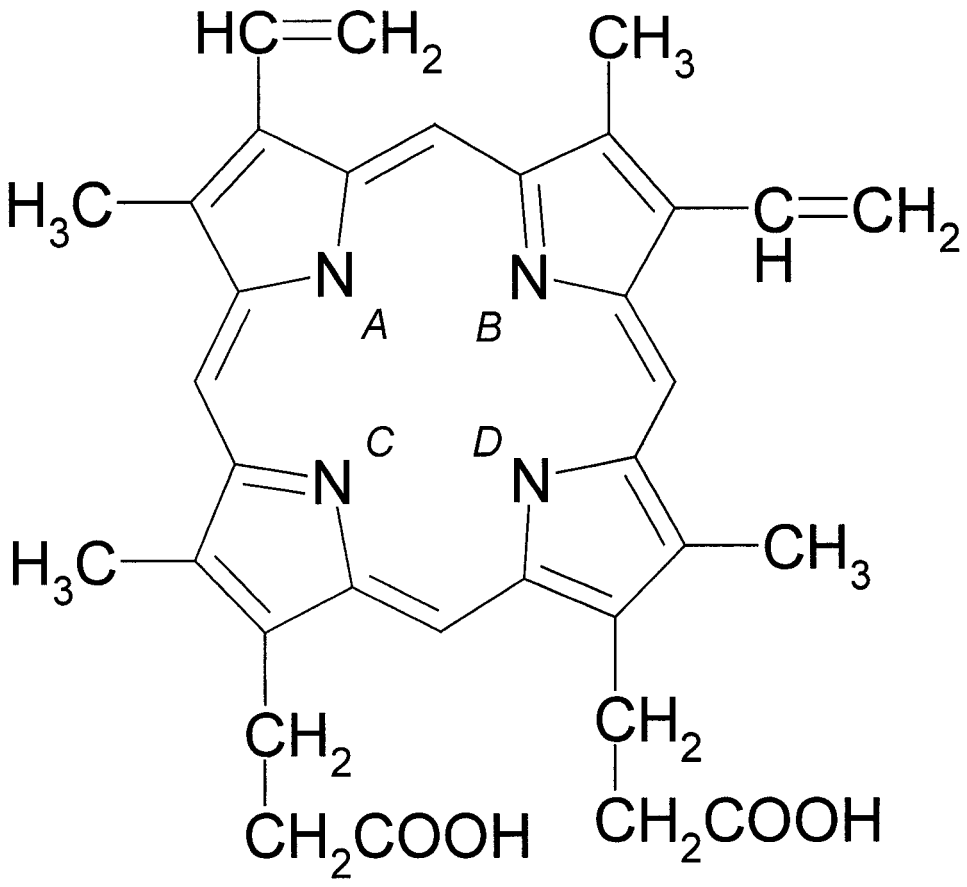
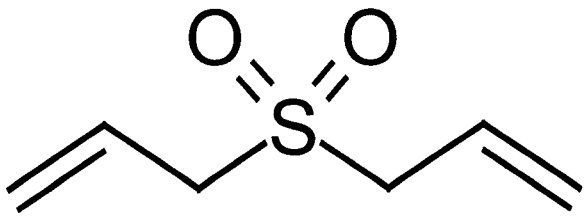


Figure 1: Structure of the heme moiety of cytochrome P450 with potential binding sites for alkylation and adduct formation labeled at the pyrrole rings (A, B, C and D). An alkyl group can bind to one of these sites, forming an *N*-alkylprotoporphyrin adduct that may result in the dissociation of the alkylated heme from the protein moiety of a cytochrome P450 enzyme and its functional deactivation.

a.



b.

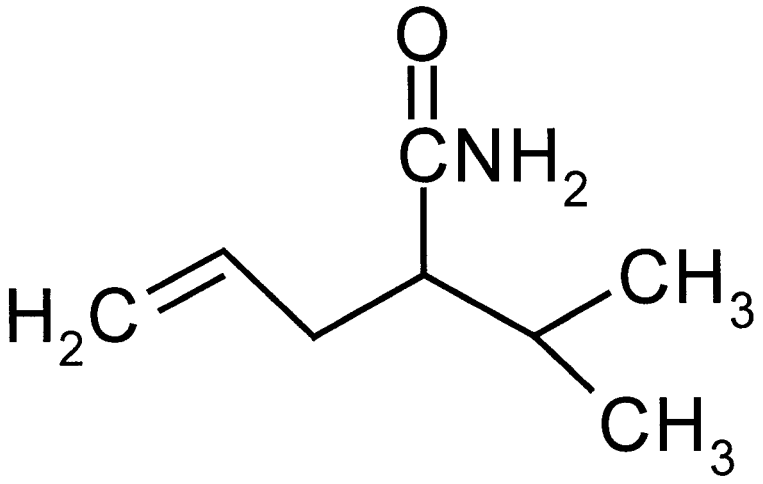


Figure 2. The chemical structures of (a) diallyl sulfone ($DASO_2$), a metabolite in garlic that has been shown to inactivate CYP2E1. (b) allylisopropylacetamide (AIA), an inactivator of CYP2C11 and CYP2B1 that is known to covalently bind to heme. Like AIA, $DASO_2$ contains terminal double bonds that are able to react with and covalently bind to other molecules, including the heme moiety of a cytochrome P450 enzyme.

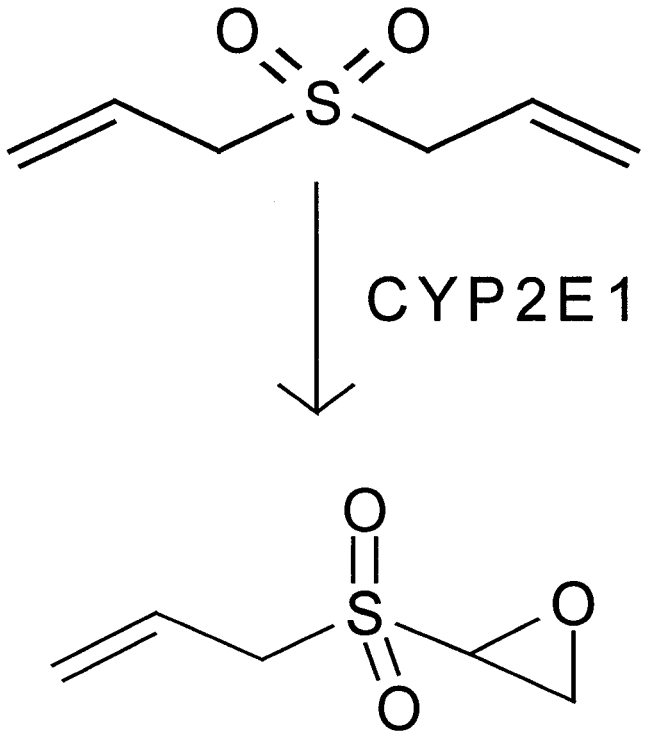


Figure 3: Proposed scheme of CYP2E1-mediated epoxidation of $DASO_2$. During the metabolism of $DASO_2$ to $DASO_3$, the heme moiety of CYP2E1 is alkylated, resulting in the formation of *N*-alkylprotoporphyrin heme adducts.

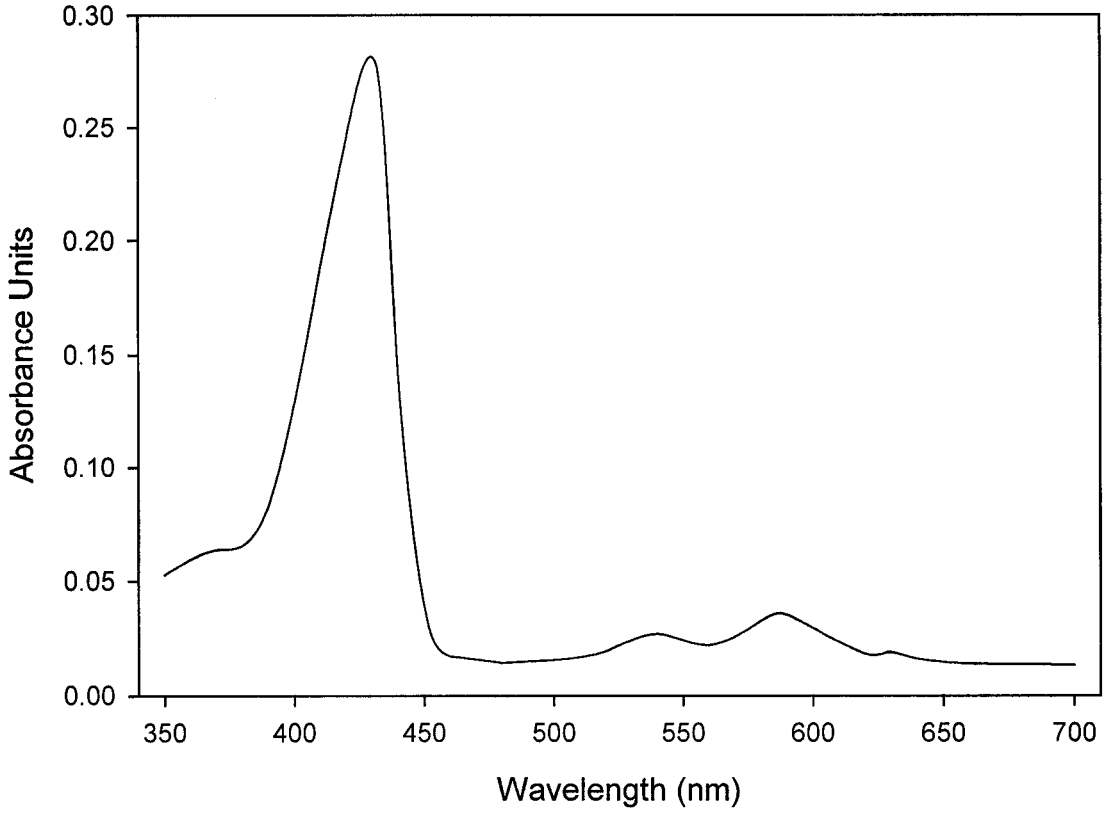


Figure 4: Sample absorbance spectrum of a zinc-complexed *N*-alkylprotoporphyrin adduct observed using UV-visible light spectrophotometry. A characteristic peak known as the Soret peak is observed at 432 nm, which can be used to quantify the amount of adducts present when divided by the molar extinction coefficient of zinc-complexed *N*-alkylprotoporphyrin adducts. Other peaks are noted at 547 nm, 591 nm and 634 nm.

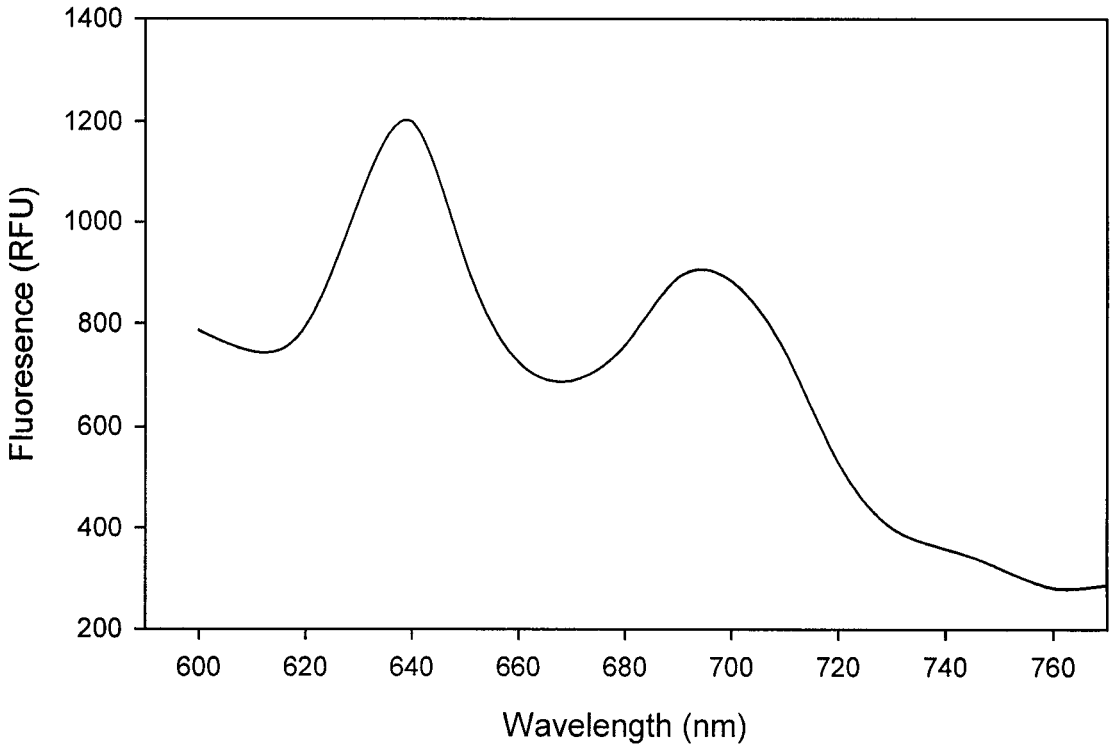


Figure 5: Sample emission spectrum of an *N*-alkylprotoporphyrin compound observed using a fluoresence plate reader with an excitation wavelength of 432 nm. Characteristic peaks are observed at 640 and 700 nm. The difference in peak wavelength from previous studies is due to differences in the chemical structure of the *N*-alkylprotoporphyrin adduct. Fluoresence is measured in relative fluoresence units, and must be compared with a standard curve to quantify the amounts of *N*-alkylprotoporphyrins formed.

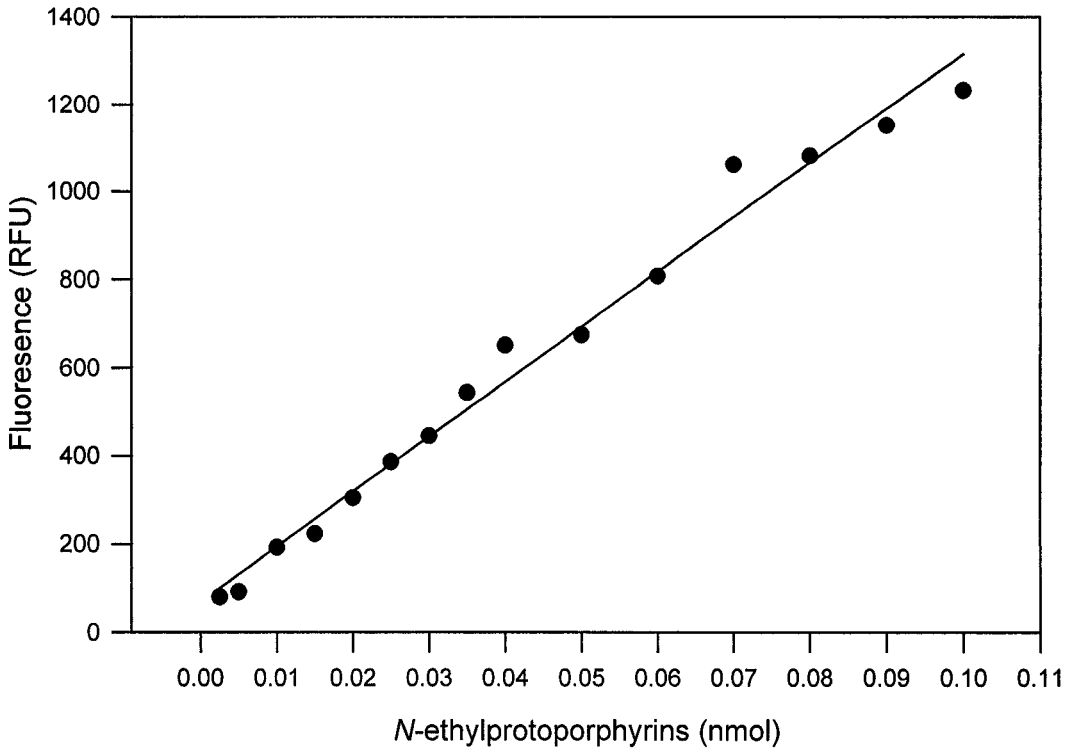


Figure 6: *N*-ethylprotoporphyrin standard curve used for the quantification of *N*-alkylprotoporphyrin adducts formed in microsomal incubations. The relationship between the amounts of *N*-ethylprotoporphyrins (nmol) and the relative fluoresence units (RFU) was linear (r^2= 0.9841).

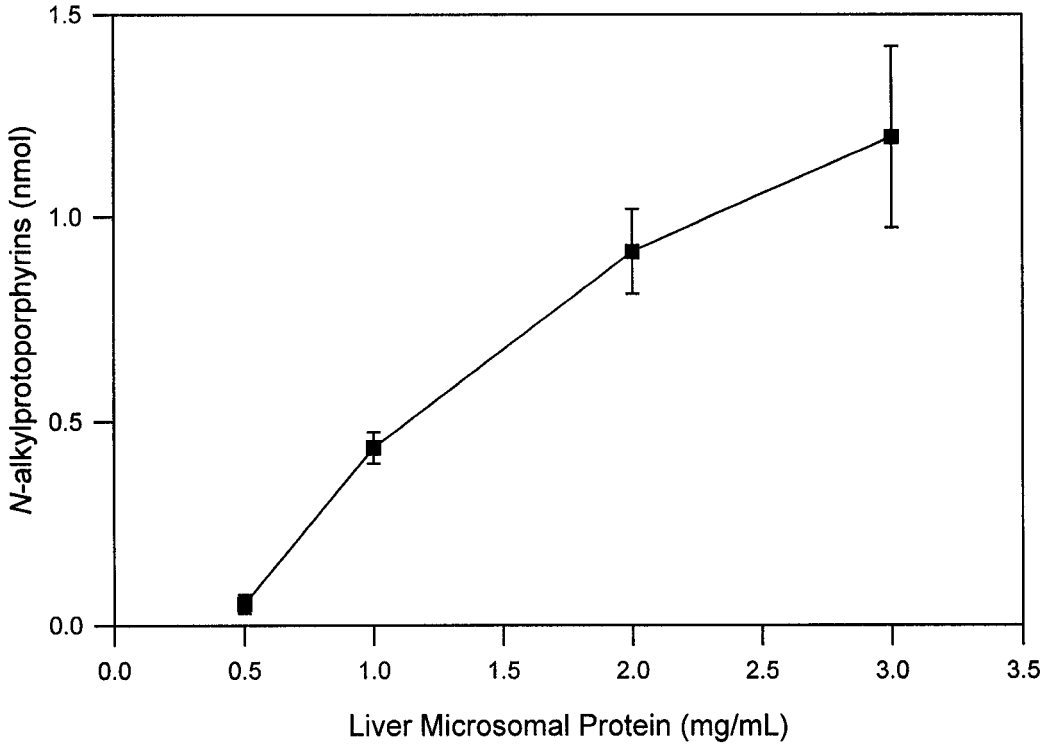


Figure 7: *N*-alkylprotoporphyrins detected in hepatic microsomal incubations with varying protein concentrations (0.5 - 3 mg/mL). Microsomes were prepared from the livers of adult male CD-1 mice. Incubations were performed with 2 mM of $DASO_2$ and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

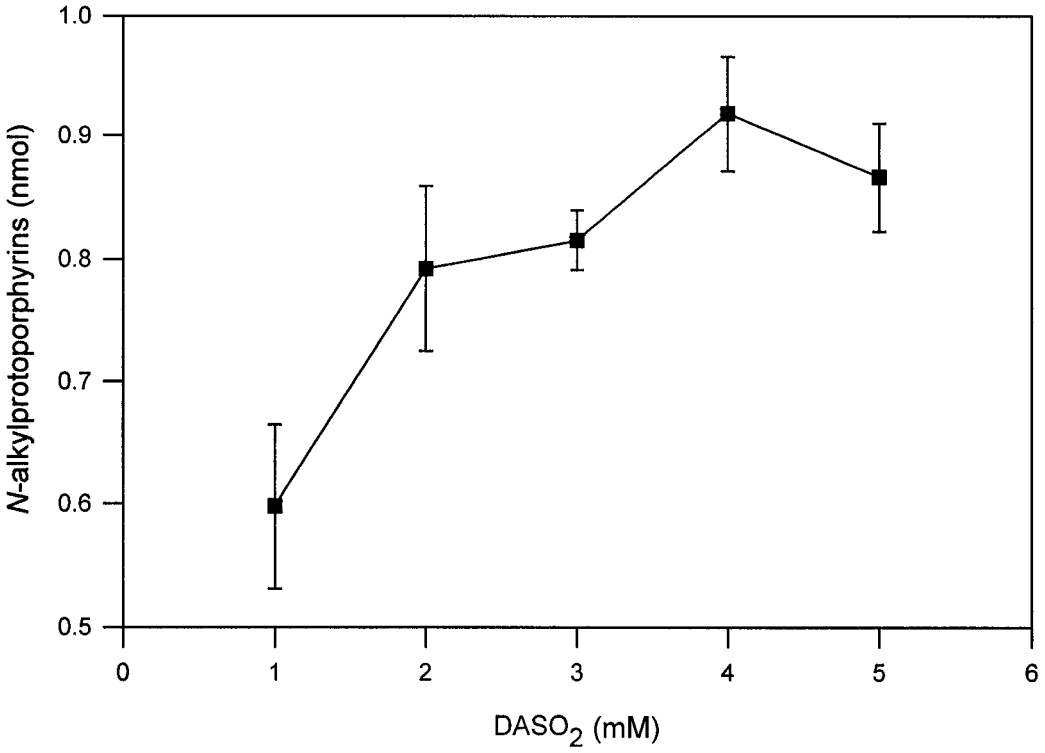


Figure 8: *N*-alkylprotoporphyrins detected in hepatic microsomal incubations with varying concentrations (1 – 5 mM) of $DASO_2$. Microsomes were prepared from the livers of adult male CD-1 mice, and incubations were performed with 3 mg/mL of protein and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

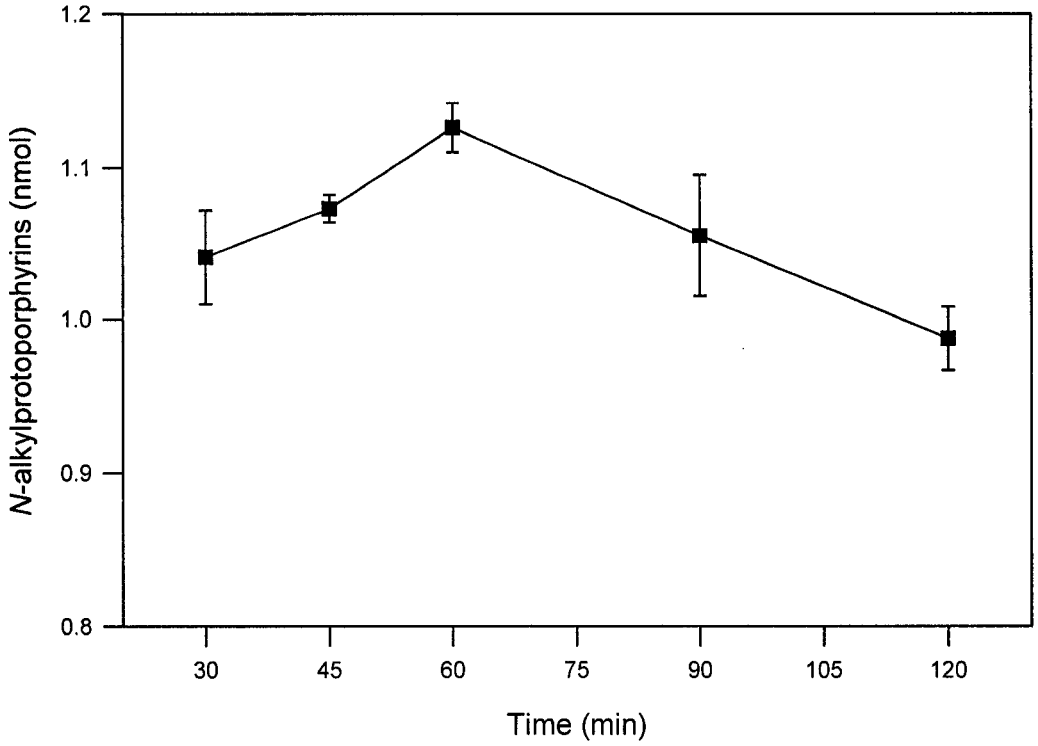


Figure 9: *N*-alkylprotoporphyrins detected in hepatic microsomal incubations carried out for 30-120 min. Microsomes were prepared from the livers of adult male CD-1 mice, and incubations were performed with 3 mg/mL of protein, 4 mM $DASO_2$ and 2.0 mM NADPH in a total volume of 1 mL at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each incubation time-point.

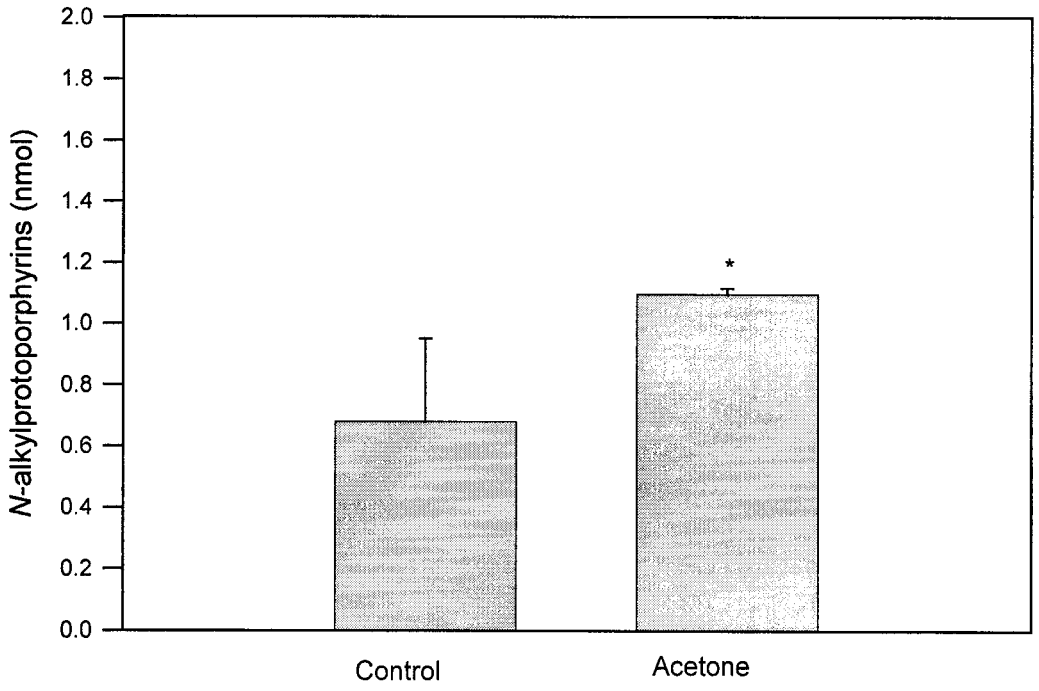


Figure 10: *N*-alkylprotoporphyrins detected in hepatic microsomal incubations from mice pretreated with acetone for 8 d and mice left untreated for the same period. Incubations were prepared with 3 mg/mL microsomal protein and 4 mM $DASO_2$ with 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations from each group. A significant difference between the groups was analyzed using the Mann-Whitney Rank Sum Test. * Significantly different from control ($p < 0.05$).

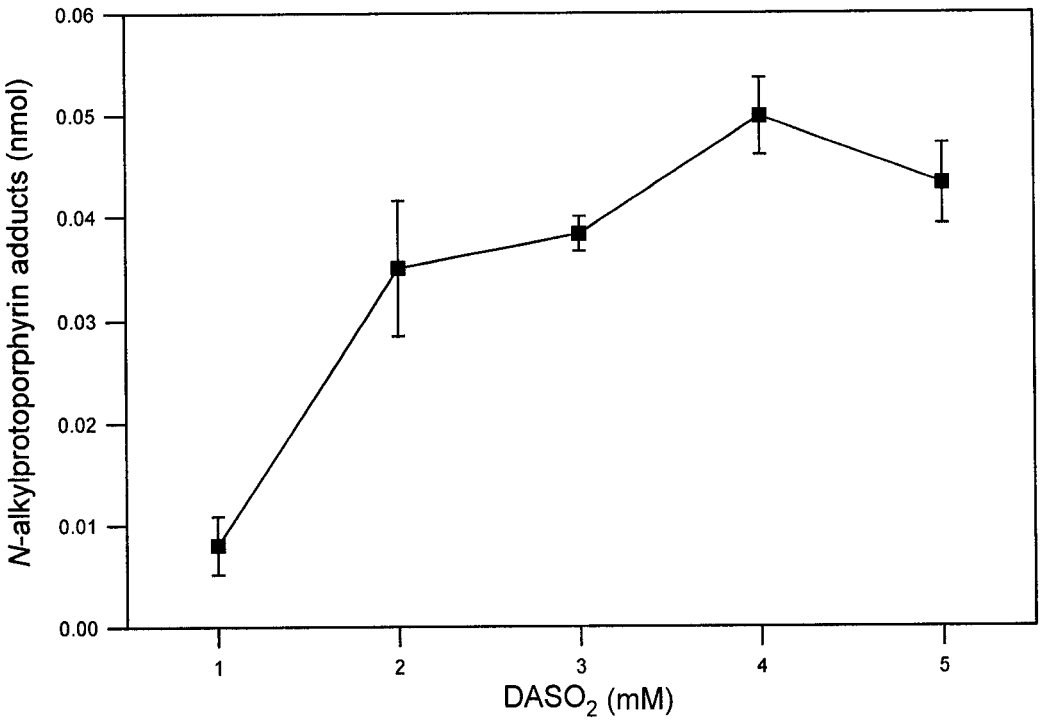


Figure 11: *N*-alkylprotoporphyrins detected in hepatic microsomal incubations performed with varying concentrations of $DASO_2$. Microsomal protein was obtained from microsomal suspensions of human liver. Incubations were performed with microsomal protein containing 150 pmol of cytochrome P450 and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

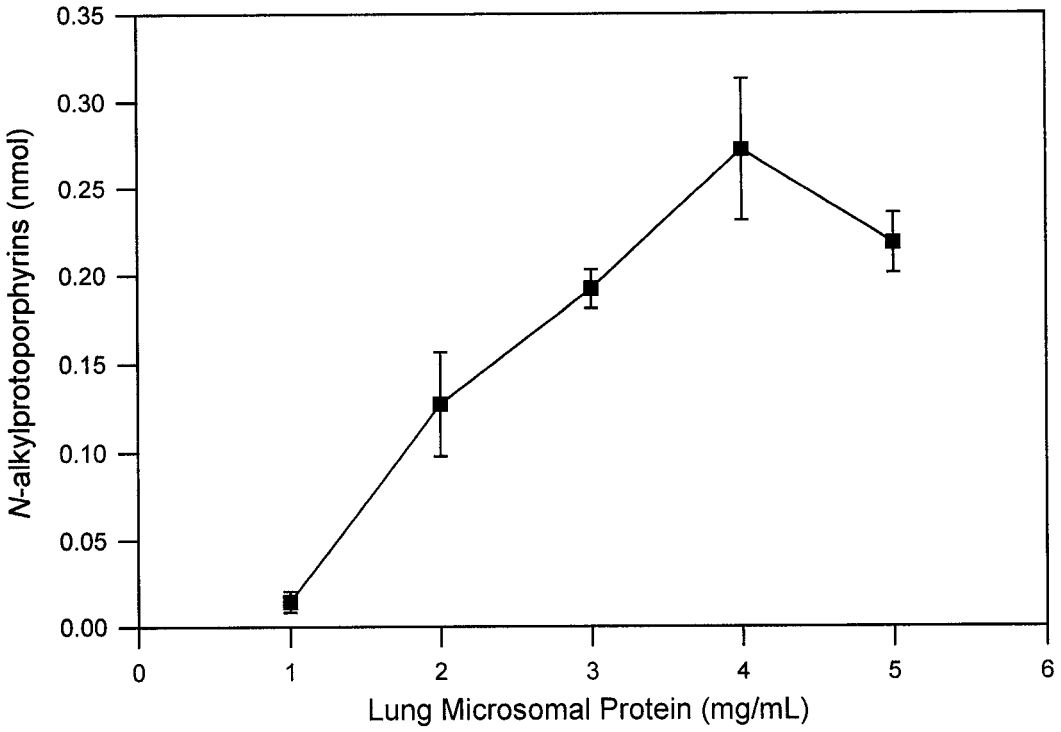


Figure 12: *N*-alkylprotoporphyrins detected in microsomal incubations containing various concentrations of microsomal protein (1 –5 mg/mL). Microsomes were prepared from the lungs of adult male CD-1 mice. Incubations were performed with 4 mM of $DASO_2$ and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

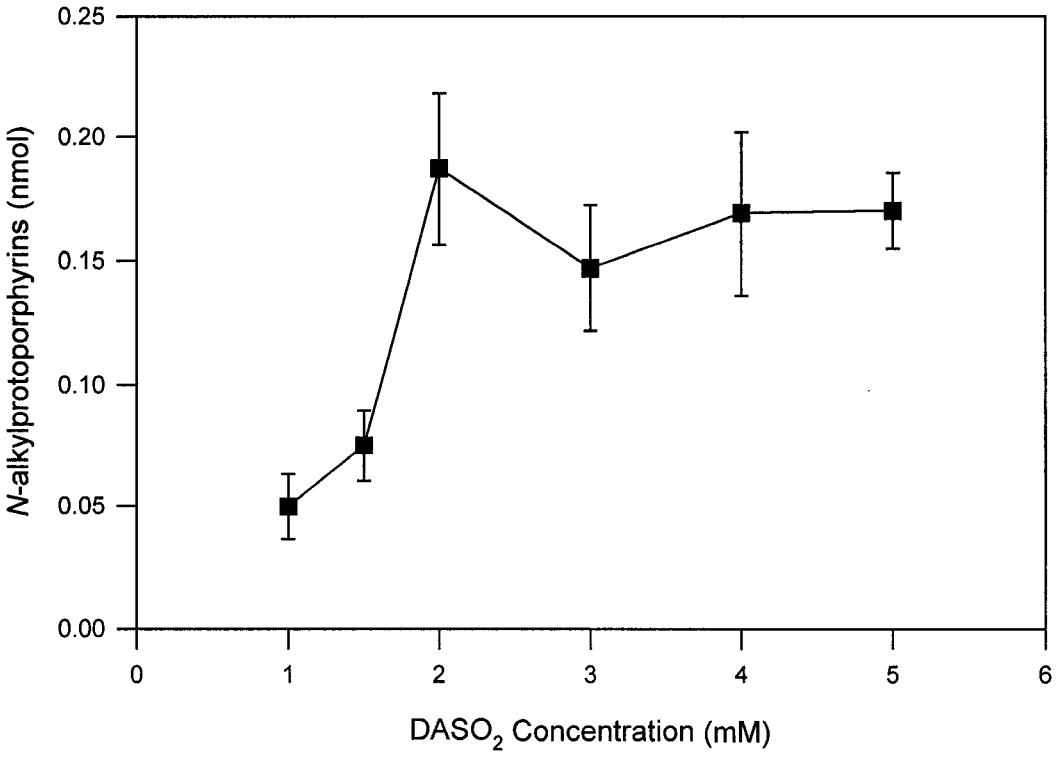


Figure 13: *N*-alkylprotoporphyrins detected in pulmonary microsomal incubations with varying concentrations (1-5 mM) of $DASO_2$. Microsomes were prepared from the lungs of adult male CD-1 mice, and incubations were performed with 5 mg/mL of protein and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

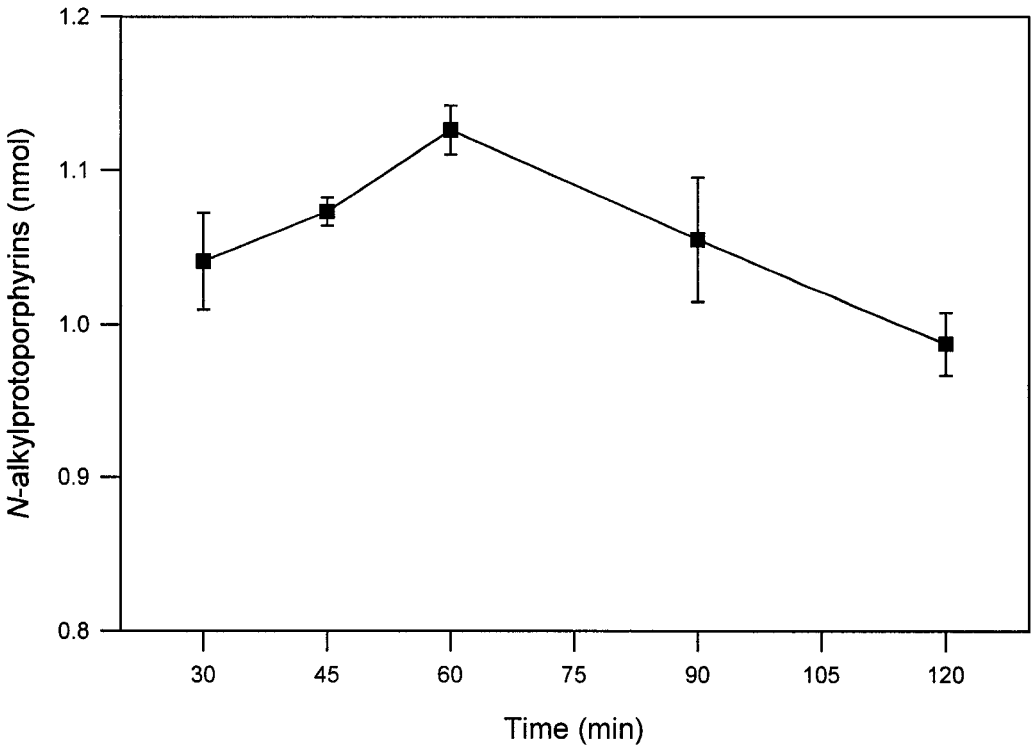


Figure 14: *N*-alkylprotoporphyrins detected in pulmonary microsomal incubations carried out for 30-120 min. Microsomes were prepared from the lungs of adult male CD-1 mice, and incubations were performed with 5 mg/mL of protein and 4 mM $DASO_2$ with 2.0 mM NADPH in a total volume of 1 mL at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each incubation time-point.

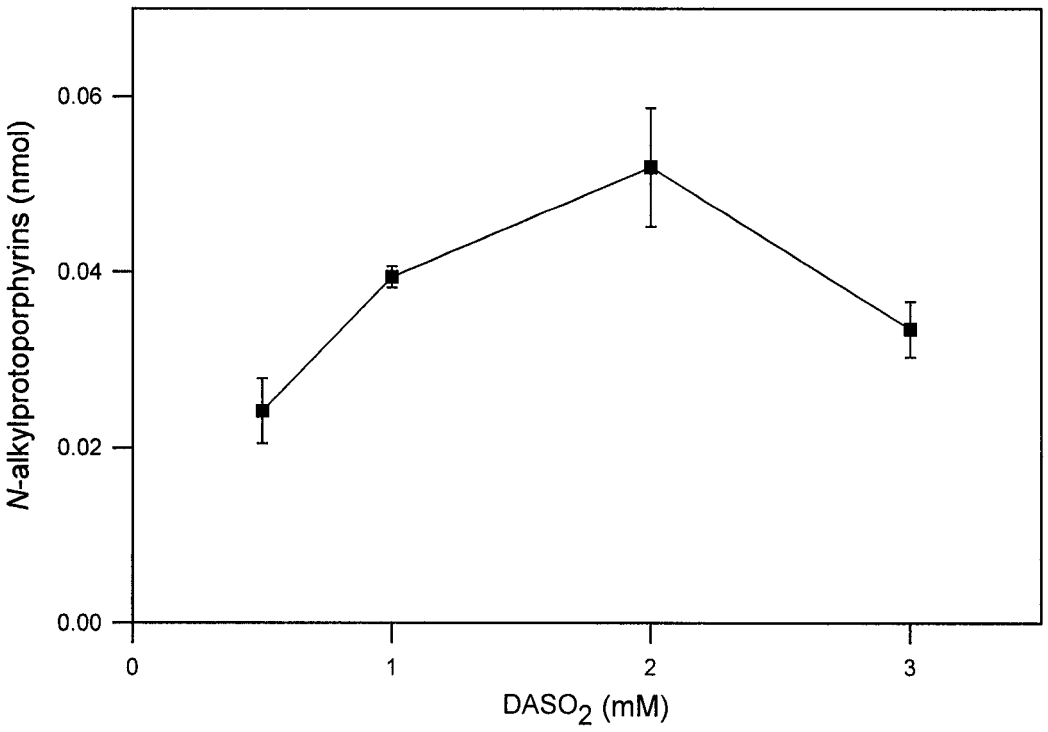


Figure 15: *N*-alkylprotoporphyrins detected in baculosome incubations with varying concentrations (0.5 - 3 mM) of $DASO_2$. Baculosomes contained rat CYP2E1-expressed microsomes. Incubations were performed with 150 pmol of cytochrome P450 in microsomal proteins and 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations at each concentration.

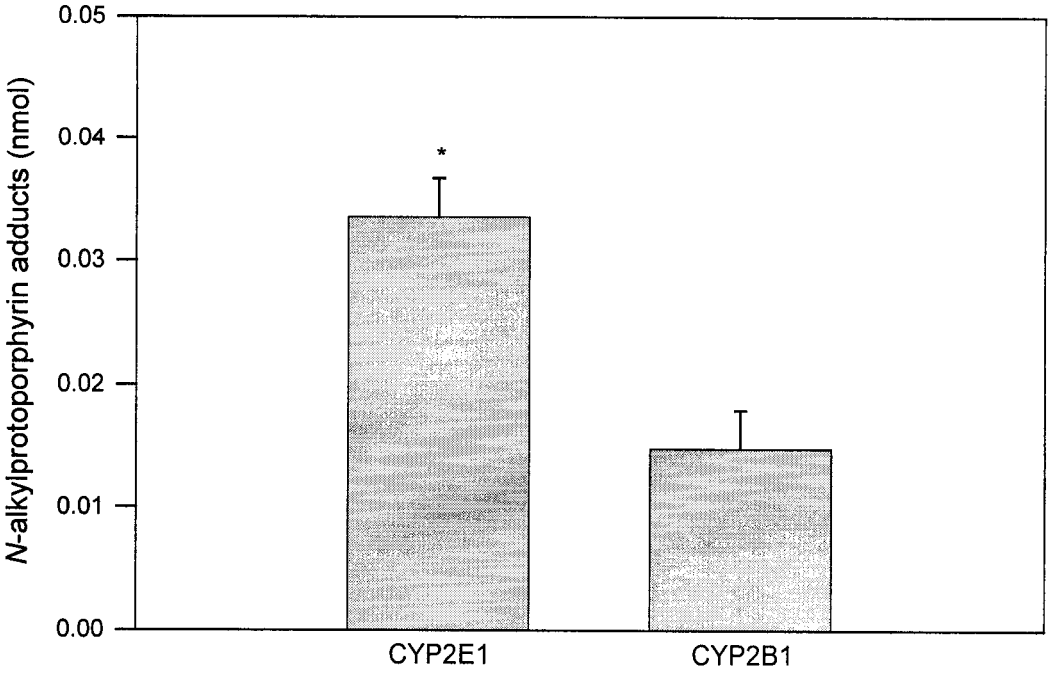


Figure 16: *N*-alkylprotoporphyrins detected in baculosomes containing rat CYP2E1- or CYP2B1-expressed microsomes. Incubations were performed with 150 pmol cytochrome P450 in microsomal proteins and 3 mM $DASO_2$ with 2.0 mM NADPH in a total volume of 1 mL for 60 min at 37°C. Data are expressed as mean ± S.D of four microsomal incubations from each group. A significant difference between the groups was analyzed using the Student's t-test. * Significantly different from the CYP2B1 incubations ($p<0.05$).

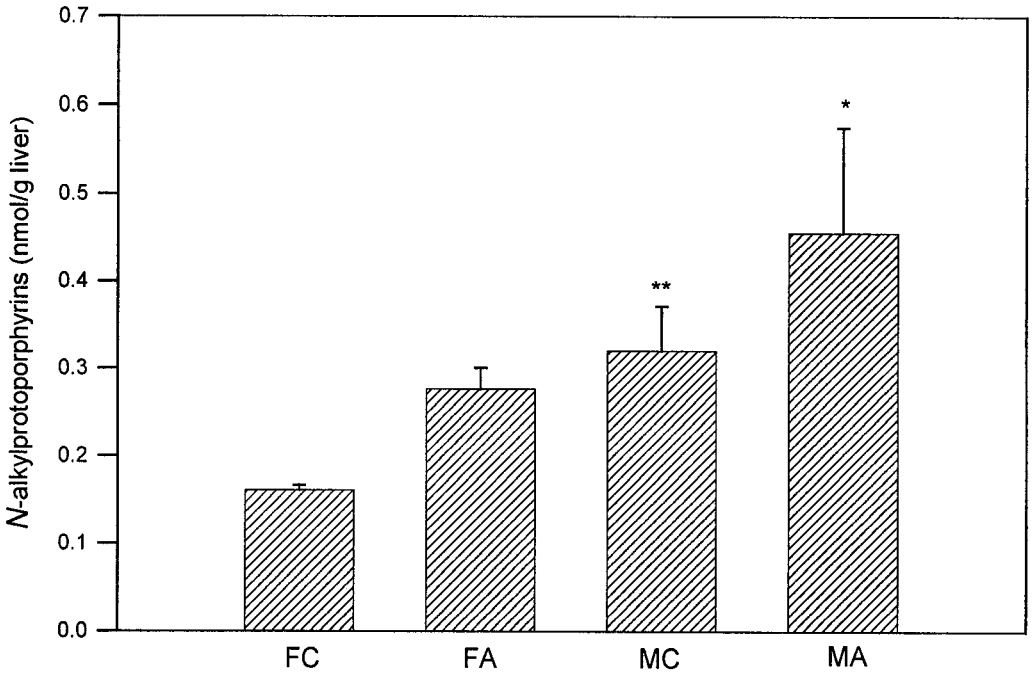


Figure 17: *N*-alkylprotoporphyrin adducts formed *in vivo* in liver of male and female mice after treatment with 100 mg/kg $DASO_2$. Data are presented as mean ± standard deviation of four tissue homogenates from each group. The data were analyzed using one-way ANOVA. Significant differences between the groups were analyzed using the Tukey test. FC – female mice, control; FA – female mice, treated with acetone for 8 d prior to sacrifice; MC- male mice, control; MA – male mice, treated with acetone for 8 d prior to sacrifice. * Significantly different from FC, FA and MC. ** Significantly different from FC. ($p < 0.05$.)

CHAPTER FOUR

DISCUSSION

This study shows that exposure to $DASO_2$ results in the formation of *N*-alkylprotoporphyrin adducts in lung and liver of mice. As expected, the levels of these adducts were higher (fivefold) in the liver than in the lung which is consistent with the higher levels of cytochrome P450 observed in the former than in the latter. The formation of these compounds was shown to be dependent on the concentration of $DASO_2$, the amounts of microsomal protein, and the duration of the incubation time. In addition, *in* vivo studies revealed that the levels of *N*-alkylprotoporphyrin adducts were higher in the livers of male versus female mice. Furthermore, *N*-alkylprotoporphyrin adducts were detected in microsomal incubations prepared from human liver. However, these adducts were not detected at quantifiable levels in microsomes prepared from human lung tissue. This phenomenon is not unexpected, as levels of cytochrome P450 are considerably lower in human lung than in human liver. However, our findings indicate that the mouse model is relevant for investigations of *N*-alkylprotoporphyrin adduct formation, at least in human liver.

It is well established that $DASO_2$ inactivates the CYP2E1 enzyme. Previous studies using murine liver microsomal incubations have shown that the catalytic activity of CYP2E1 as assessed by PNP hydroxylation was reduced by 38% after incubation with $DASO_2$ (Premdas et al. 2000). Levels of immunodetectable CYP2E1 were also reduced after exposure to $DASO_2$ (Premdas et al. 2000). Brady et al. (1991) found that after treating rats with DAS for 4 weeks, NDMA demethylation, a measure of CYP2E1 catalytic activity, was decreased, as were the amounts of immunodetectable CYP2E1

protein. DASO and $DASO_2$, the metabolic products of DAS oxidation, were also observed in rat blood, urine and liver after treatment with DAS, suggesting that these compounds were derived from the CYP2E1 metabolism of DAS. Numerous studies have shown that treatment with $DASO_2$ or its parent compounds prevent toxicity by xenobiotics that are metabolized by CYP2E1, inhibiting the toxic effects of such toxicants as DCE and TCE (Yang et al. 1994, Forkert et al. 1996b, Forkert et al. 2002). Forkert et al. (2000) reported that elevated formation of the epoxide $DASO_3$ was observed after treatment with $DASO_2$. This reaction is concentration-dependent, and is catalyzed by CYP2E1. Furthermore, there were decreases in heme content and in CYP2E1 catalytic activity (Premdas et al. 2000). These studies demonstrated that the formation of the epoxide is related to the loss of CYP2E1 function, prompting speculation that $DASO_3$ may be the reactive species that binds to the heme molecule and forms the *N*-alkylprotoporphyrin adduct. However, studies with other compounds have suggested that epoxides may not be directly involved in the inactivation of cytochrome P450, and that a reactive intermediate formed during the epoxidation reaction is responsible for alkylating the heme moiety. Studies with AIA, 2-isopropyl-4-pentenoate and novonol have shown that neither the epoxides of these compounds formed by their cytochrome P450 targets nor their secondary metabolites play a direct role in heme alkylation and cytochrome P450. Instead, a reactive cationic intermediate molecule formed during the epoxidation reaction may alkylate the heme moiety (Ortiz de Montellano et al. 1979, 1984). To fully characterize the role of the $DASO_3$ epoxide in the formation of *N*-alkylprotoporphyrin adducts, the chemical structure of the adduct must be determined. Attempts to use NMR spectroscopy in these experiments failed, possibly due to the small amounts of compound obtained and the high molecular weight of the heme molecule. However, based upon these observations, it is

plausible that $DASO_2$ alkylates the heme via the formation of reactive cationic intermediates during the epoxidation reaction mediated by CYP2E1, resulting in the production of *N*-alkylprotoporphyrin adducts.

The formation of *N*-alkylprotoporphyrin adducts is related to a decrease in observable heme content. In mouse liver microsomes, administration of $DASO_2$ resulted in a 70% decrease of heme levels (Premdas et al. 2000). The decrease in heme is similar to that of other porphyrinogenic compounds that alkylate the heme moieties of cytochrome P450 enzymes, such as AIA. In the present experiments, we have shown that *N*-alkylprotoporphyrin adducts were formed after exposure to $DASO_2$. The similarity of activity to compounds such as AIA suggests that $DASO_2$ is a porphyrinogenic compound that alkylates the heme moiety of CYP2E1. The mechanism for this alkylation in AIA is well established. The terminal double bonds of AIA react with a nitrogen atom on one of the four pyrrole rings of CYP2C11, forming a covalent bond that results in the formation of *N*-AIA protoporphyrin IX (Wong and Marks 1999). Augusto et al. (1982) suggested that cytochrome P450 oxidizes xenobiotics such as 4-ethyl DDC to form a radical cation, which is then cleaved from the xenobiotic and alkylates the heme molecule. Bornheim et al. (1987) found that the heme adduct then dissociates from the apoprotein but lea19ves it intact, allowing heme from regulatory reserves to reconstitute the functional enzyme but resulting in a depletion of free heme. Like AIA, $DASO_2$ contains terminal double bonds that act as reactive sites for metabolism and possible covalent binding to heme. Forkert et al. (2000) suggested that the terminal double bonds acted as the reactive site for CYP2E1-mediated epoxidation. Our data suggest that alkylation of the heme moiety and the formation of the *N*-alkylprotoporphyrin adduct occur after the oxidation of these bonds by CYP2E1 and the subsequent formation of $DASO_3$.

The observation of *N*-alkylprotoporphyrin adducts in our study required the use of various detection methods. Because the *in vivo* studies used homogenates of tissue without the extraction and isolation of microsomal proteins, the methodology left the possibility that other compounds and metabolites formed through other metabolic processes may obscure detection of the *N*-alkylprotoporphyrin adducts. As well, thin-layer chromatography does not represent an optimal method of detecting heme adducts. Wong and Marks (1999) reported that *in vivo* protocols resulted in a 25% recovery of *N*-alkylprotoporphyrin adducts, as some sample was lost to the TLC plates during preparation. However, *in vivo* treatments also result in a twofold increase in the amount of cytochrome P450 affected by porphyrinogenic compounds when compared to levels in the *in vitro* system. (Ortiz de Montellano and Mico 1981). This may be due to the replenishing activity of heme in the body, which may reconstitute the active enzyme, as well as the variety of cytochrome P450 enzymes and hemoproteins in tissues ***in vivo*** that may provide additional sources of alkylated heme adducts (Ortiz de Montellano and Mico 1981). Experiments in this study used a combination of methods. Thin-layer chromatography and UV-visible spectrophotometry were used for *in vivo* experiments, as the samples were deemed large enough to remain quantifiable even after loss of sample to the TLC plate. Fluorometry has been described as a more sensitive method for *N*-alkylprotoporphyrin detection, particularly under *in vitro* conditions (Lavigne et al. 2002). This allowed for the detection of the smaller amounts of adducts formed in microsomal incubations without the loss of sample accompanying the TLC method. In our studies, the use of a fluorescence plate reader allowed for the detection of small amounts of adducts suspended in low volumes of solvent, facilitating the detection of heme adducts that would not be seen in the greater dilutions necessary for the standard fluorometric method.

However, fluorometry was not appropriate for *in vivo* quantification, as TLC allowed for identification and enrichment of the red fluorescent band. Samples that were not purified using TLC were too impure to analyze using the fluorometric method, with other proteins and homogenate residues obscuring the *N*-alkylprotoporphyrin adducts.

Previous studies have clearly shown that $DASO_2$ and its parent compounds act as inhibitors of CYP2E1 (Brady et al. 1991, Yang et al. 1994, Forkert et al. 2000). The data from our experiments show that CYP2E1 is involved in the formation of *N*-alkylprotoporphyrin adducts after administration of $DASO_2$, suggesting the formation of these adducts to be a significant event in inactivation of CYP2E1 by $DASO_2$. Baculosome incubations expressing rat CYP2E1 resulted in adduct formation when exposed to $DASO_2$. The effects of acetone pretreatment on the formation of *N*-alkylprotoporphyrin adducts also provide evidence for the involvement of CYP2E1. In previous studies, mice pretreated with acetone for 8 d showed a 5.3-fold increase in the amounts of CYP2E1 (Forkert et al. 1994). In this study, microsomes prepared from acetone-treated mice formed 30% more adducts than microsomes from untreated mice when incubated with $DASO_2$. As well, formation of *N*-alkylprotoporphyrin adducts was greater in the livers of acetone-treated mice than in the lung. This phenomenon can be ascribed to the finding that acetone induces CYP2E1 in liver, but not in lung. The increase in the formation of *N*-alkylprotoporphyrin adducts after treatment with acetone is similar to that reported for with the activity of AIA; male rats treated with PB for 4 d prior to AIA treatment showed a significant elevation in the formation of *N*-AIA protoporphyrin adducts (Lavigne et al. 2002). This is consistent with the assumption that PB acts as an inducer for the CYP2B1/2 enzymes, which provides more enzyme for inactivation by AIA (Lavigne et al 2002). A similar mechanism is likely in the case of $DASO_2$ and acetone pretreatment.

Acetone is a known inducer of CYP2E1 (Forkert et al. 1994) and results in an increased amount of the enzyme available for reaction. These results support the premise that CYP2E1 is the primary target for inactivation caused by $DASO_2$.

Acetone may also induce or inhibit other cytochrome P450 enzymes, meaning that our results may reflect a potential for $DASO_2$ to inhibit cytochrome P450 enzymes other than CYP2E1. This assertion is supported by experiments that showed that *N*-alkylprotoporphyrins were formed in baculosome incubations containing rat-expressed CYP2B1 as well as those containing rat-expressed CYP2E1. Lavigne et al. (2002) suggested that 70% of the *N*-alkylprotoporphyrins formed by AIA originated from the heme moiety of CYP2C11 in male rats, while the remaining 30% originated from other enzymes, such as CYP2B1. Other heme-containing proteins may also be available for reaction, such as cytochrome b_5 (Premdas et al. 2000). This assumption is further supported by the *in vivo* data, which showed that the amounts of *N*-alkylprotoporphyrins formed in the livers of female mice was lower than those observed in male mice, even though the levels of CYP2E1 show no gender-difference in liver tissue (Hong et al. 1987). It may be that other cytochrome P450 enzymes with differences in expression between genders, such as CYP2B1, are inactivated by $DASO_2$, resulting in a higher amount of heme adducts in males than in females. Acetone induction resulted in an increase in the amounts of *N*-alkylprotoporphyrins observed; while the increase was not statistically significant in females, a significant increase was observed in male liver tissues after treatment with acetone. This suggests that with more CYP2E1 available for inactivation, more heme adducts will be formed, adding credence to the assumption that CYP2E1 remains the primary target of $DASO_2$–mediated inactivation. It is likely,

therefore, that $DASO_2$ acts as an inhibitor of other cytochromes, though the major source of *N*-alkylprotoporphyrins appears to be CYP2E1.

Our data have shown that $DASO_2$ inactivates CYP2E1 by alkylating the heme moiety and forming *N*-alkylprotoporphyrin adducts, but other mechanisms for CYP2E1 inactivation exist. Three distinct mechanisms for suicidal inactivation of cytochrome P450 enzymes have been observed. Halpert and Neil (1981) showed that the organophosphate compound, parathion, was able to inactivate cytochrome P450 by forming a covalent bond between the sulfur atom of parathion and the apoprotein of cytochrome P450. Ortiz de Montellano and Correia (1983) showed that chemical compounds can bind irreversibly to the prosthetic heme group, decreasing the catalytic activity of the enzyme and destabilizing the apoprotein. A third mechanism occurs when a metabolite of a compound such as 4-ethyl DDC alkylates the heme molecule, which then fragments and binds to the apoprotein, causing the destruction of the protein moiety (Davies et al. 1986, Correia et al 1987). Some cytochrome P450 inactivation may be based upon a combination of two or more of these mechanisms. Davies et al. (1986) suggested that AIA-mediated heme alkylation accounted for the destruction of only 60% of the cytochrome P450 complement in rat liver, and that a further 28% could be destroyed by the binding of heme degradation products to the apoprotein. As well, only 62% of the inactivated enzyme could be restored by reconstitution with exogenous heme.

The data from our experiments confirm that heme alkylation is observed in the inactivation of CYP2E1 by $DASO_2$, but these findings do not preclude further inactivation of the apoprotein by heme degradation product binding as a secondary metabolic event. Premdas et al. (2000) showed that the inactivation of CYP2E1 by $DASO_2$ was accompanied by the loss of immunodetectable CYP2E1 and a dose-

dependent reduction in the catalytic activity of CYP2E1. This suggests that modification of the apoprotein may also play a role in the inactivation of CYP2E1, though our data suggest that heme alkylation remains a major event in CYP2E1 inactivation by $DASO_2$.

Summary and Conclusions

These experiments confirm that $DASO_2$ inactivates CYP2E1 by binding to the heme moiety of the enzyme, alkylating one of the pyrrole rings and preventing the enzyme from biotransforming other substrates. *N*-alkylprotoporphyrin adducts are observed after the administration of $DASO_2$ in murine lung and liver, in baculosomes containing rat CYP2E1-expressed microsomes, and in microsomes prepared from human liver. Pretreatment with acetone increases the formation of these adducts, supporting the assertion that CYP2E1 is the enzyme involved in *N*-alkylprotoporphyrin formation. This finding was confirmed with the detection of N-alkylprotoporphyrins in baculosomes incubations that expressed CYP2E1. Our experiments have shown that $DASO_2$ inactivates the heme moiety of CYP2E1 in a manner that may explain its inhibitory effects and its potential for preventing xenobiotic-mediated toxicity in the liver and lung.

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