

2004

## Exposures to silica and inducers of xenobiotic metabolism in the rat lung

Lori A. Battelli  
*West Virginia University*

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Exposures to Silica and Inducers of Xenobiotic  
Metabolism in the Rat Lung

Lori A. Battelli

Thesis submitted to the  
Davis College of Agriculture, Forestry, and Consumer Sciences  
at West Virginia University in partial fulfillment  
for the degree of

Master of Science  
in  
Genetics and Developmental Biology Program

Ann Hubbs, Ph.D. Chair  
Joginder Nath, Ph.D. Co-Chair  
Sharon Wenger, Ph.D.

Morgantown, WV  
2004

Keywords: Cytochrome P4501A1, polycyclic aromatic hydrocarbons,  
respirable particles, silica, epidemiology

## ABSTRACT

### EXPOSURES TO SILICA AND INDUCERS OF XENOBIOTIC METABOLISM IN THE RAT LUNG

Lori A. Battelli

CYP1A1 metabolizes polycyclic aromatic hydrocarbons (PAHs), such as those in cigarettes, to reactive intermediates which interact with DNA and lead to cancer. Silica is a lung carcinogen. Epidemiology studies of silica and lung cancer are not all positive. A possible explanation for these inconsistencies may be that silica is a modifier of PAH metabolism and, thus, cigarette smoke carcinogenesis. We hypothesize that crystalline silica exposure alters CYP1A1 expression, thereby modifying lung cancer risk. Rats exposed to both crystalline silica and the model PAH,  $\beta$ -naphthoflavone (NF), a CYP1A1 inducer have significantly decreased CYP1A1 enzymatic activity and CYP1A1 protein expression. In the proximal alveolar region of NF-exposed rats, silica exposure increases markers of alveolar type II cells but decreases proportional CYP1A1 expression in type II cells as detected by immunofluorescence. Our experiments support the hypothesis that silica is a negative modifier of CYP1A1 induction by PAH.

## ACKNOWLEDGEMENTS

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I must first express my gratefulness for the continuous moral support and guidance from Dr. Ann Hubbs. I also thank Dr. Joginder Nath and Dr. Sharon Wenger for participating on my committee and providing valuable insight.

There are many people at NIOSH that I am grateful for their help and support: Mark Barger, for assisting in IT installations, lung micrososome preparation, and Western Blot technique.

Mike Kashon for statistical analysis, Mike Whitmer and Dan Lewis for endotoxin analysis, and Val Vallyathan for providing silica particles and analysis data.

The PPRB histology lab for preparing the microscope slides. Patsy Willard for her valuable advice on IHC and IF staining and Lyndell Millecchia for photomicroscopy and imaging support.

Dr. Mohamed Ghanem for advice, support and help in conducting experiments.

There are also many friends and family members I must also thank for their encouragement and support of my graduate work.

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Ahr	Aryl hydrocarbon receptor
AIP	Aryl hydrocarbon receptor interacting protein
AM	Alveolar macrophage
Arnt	Aryl hydrocarbon receptor nuclear translocator
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic Acid
Bhlh	Basic helix loop helix
BSA	Bovine serum albumin
CYP	Cytochrome P450
CYP1A1	Cytochrome P4501A1
CYP2B1	Cytochrome P4502B1
EDTA	Ethylenediamine tetracetate
ECOD	7-Ethoxycoumarin- <i>O</i> -deethylase
EROD	7-Ethoxyresorufin- <i>O</i> -deethylase
H & E	Hematoxylin and Eosin
HRP	Horse radish peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IARC	International Agency for Research on Cancer
IP	Intraperitoneal
IT	Intratracheal
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
NF	$\beta$ -naphthoflavone
NFKB	Nuclear Factor Kappa B
PAH	Polycyclic aromatic hydrocarbon
PAS	Pers-arnt-sim
PBS	Phosphate Buffered Saline
PROD	7-Pentoxyresorufin- <i>O</i> -deethylase
SDS	Sodium dodecyl sulphate
TBS	Tris Buffered saline
TNF $\alpha$	Tumor necrosis factor alpha
XRE	Xenobiotic response element



## Chapter 1

### Introduction

Cytochrome P450s (CYPS) have been shown to play a key role in activating procarcinogens. Cytochrome P4501A1 (CYP1A1) is responsible for the metabolism of polycyclic aromatic hydrocarbons (PAHs), such as those found in tobacco byproducts. In the early 1970s, cultured lymphocytes showed a link between high AHH (aryl hydrocarbon hydroxylase) activity and increased risk of lung cancer (Kellerman *et al*, 1973). CYP1A1 is an important enzyme involved in lung cancer initiation because it activates PAHs and is responsible for the expression of the AHH activity (Bartsch *et al* 1992; Bartsch *et al*,1999; Kellerman *et al*, 1973). Experiments have shown that during CYP1A1 metabolism, PAHs are epoxidized, then hydrolyzed by epoxide hydrolase to a dihydrodiol and ultimately oxidized to the diolepoxide or ultimate carcinogen (Hasler *et al*, 1999). These diolepoxides are highly reactive and can form DNA adducts leading to mutations and possibly activating oncogenes or tumor suppressor genes (Kondraganti, *et al*, 2002).

Humans are continuously exposed to a variety of foreign compounds via lung inhalation. These exposures can lead to alterations in the lung metabolism or cause cellular injury, or both. Workers in both mining and non-mining (masonry, pottery, sand blasting) professions can be exposed to respirable silica. Exposures to respirable silica can be both chronic and acute and can lead to silicosis, and related diseases such as silicotuberculosis, and scleroderma. Although there have been inconsistencies noted in data associating risk of lung cancer and exposure to respirable silica, the International Agency for Research on Cancer (IARC) classified silica as a carcinogen in 1997. Cocco

*et al* (2001) found an association between lung cancer and silica exposure that surprisingly was stronger in workers exposed to silica than workers exposed to both silica and PAHs. PAHs, such as those found in cigarette smoke are known to increase the risk of developing cancer. Knowing that both silica exposure and PAH exposure alone can increase lung cancer risk one might expect an additive effect when co-exposed. However, if silica modifies the carcinogenicity of the PAHs, these two lung carcinogens are not simple co-variables in causing lung cancers, but instead represent a complex risk. Experiments by Miles *et al* (1993; 1994) and Levy *et al* (1997) investigated the possible link between silicosis and lung cancer and more specifically, the possible role of the pulmonary microsomal cytochrome P450 system in the initiation of lung cancer. In both studies, rats were intratracheally exposed to silica dust. Miles *et al* showed that the pulmonary microsomal CYP system was altered by intratracheal silica exposure resulting in increased activity levels of both CYP2B1 and CYP1A1 (Miles *et al*, 1993; Miles *et al*, 1994). Immunohistochemical (IHC) staining in lung tissues was negative for CYP1A1 expression in any cells. CYP2B1 was found in Clara cells and alveolar type II cells, but was not significantly different between groups (Levy *et al*, 1997).

It is important to note that those experiments were done in lung that did not have CYP1A1 induced by PAH exposure. Immunohistochemical experiments by Levy *et al* (1997) showed that the increase in CYP activity was not due to the proliferation of type II cells of silicosis. In addition, the major known effect of CYP1A1 is on the metabolism of PAHs. Since PAHs both induce and are metabolized by CYP1A1, the effect of silica on CYP1A1 is most important where there are also exposures to PAHs.

Based on those previous experiments, our experiments involve the use of CYP inducers and IT silica exposure. In the first experiment, we hypothesized that the new type II cells of silicosis were a potential site for CYP1A1 induction in the rat lung. Rats were exposed to silica and inducers of CYP –  $\beta$ -naphthoflavone, a CYP1A1 inducer; phenobarbital, a CYP2B1 inducer; or a combination of both. Endpoints measured included IHC for both CYP1A1 and CYP-dependent xenobiotic activity (7-ethoxyresorufin-*O*-deethylase (EROD) and 7-ethoxycoumarin-*O*-deethylase (ECOD) respectively).

Based on results from the first experiment, we found it necessary to use immunofluorescence (IF), more specifically dual immunofluorescence to further determine the location of CYP1A1 in the lung. IF was selected because it is more sensitive than enzymatic IHC and easily allows for double labeling the tissue for 2 different proteins. Images were digitally captured and changes quantified by morphometric analysis. The sites of CYP isoform localization were determined by measuring colocalization of a CYP1A1 fluorescent probe with a marker of a specific cell type. In addition, the total area of the lung involved in CYP1A1 expression were measured by use of these fluorescent probes and the percent of that area of expression localized to alveolar type II cells was calculated from the co-localization of CYP1A1 and cytokeratin 8, an alveolar type II cell marker. Again, rats were exposed to silica and the CYP1A1 inducer  $\beta$ -naphthoflavone. Endpoints included IF for CYP1A1 and cytokeratin 8, Western blot for CYP1A1, CYP1A1-dependent activity (EROD) and CYP2B1 dependent activity (7-pentoxoresorufin-*O*-deethylase (PROD)).

We also examined the hypothesis that the reduction of CYP1A1 activity by silica exposure was dose responsive, i.e., does this effect occur at low exposures and increase with increasing exposures. All animals were induced with NF and IT exposed to 5, 10 or 20 milligrams of silica. Endpoints measured included CYP1A1 dependent activity (EROD), CYP2B1 dependent activity (PROD), and Western blots for both CYP1A1 and CYP2B1.

## Objectives

### Study objectives:

1. **Determine the relationship between co-exposure to respirable silica dust and PAHs and CYP1A1 expression.**
2. **Determine the relationship between silicotic histopathologic lung changes and the pattern of CYP1A1 expression in alveolar epithelial cells.**

## Chapter 2

### Review of Literature

#### 1 - Cytochrome P450 (CYPs)

Cytochrome P4501A1 is a part of the Cytochrome P450 superfamily. CYPs are hemoproteins that are part of an enzyme system that help mediate xenobiotic metabolism. In the late 1950s, Klingenberg discovered this unique protein that was known as “microsomal carbon monoxide-binding pigment” (Omura, 1999; Estabrook, 1998). Omura and Sato discovered the hemoprotein nature of this pigment and proposed the name Cytochrome P450 due to its maximum optical absorption peak of 450 nm (Omura, 1999; Estabrook, 1998). In this same time period (early 1960s) Hashimoto, Yamano, and Mason discovered what they thought was a new compound in liver microsomes. It turned out to be identical to Cytochrome P450 (Omura, 1999). The exact nature and function of this hemoprotein was discovered in 1963. Estabrook, Cooper, and Rosenthal were able to confirm that Cytochrome P450 was involved in hydroxylation reactions in 1963 (Omura, 1999). More experiments followed and Cytochrome P450 was found to be a mixed function oxidase, providing molecular oxygen to these reactions.

The body is constantly exposed to foreign compounds and the majority of these compounds are metabolized through the cytochrome P450 system (Philpot and Smith, 1984). There are two phases to these reactions, and CYPs participate in Phase I reactions.

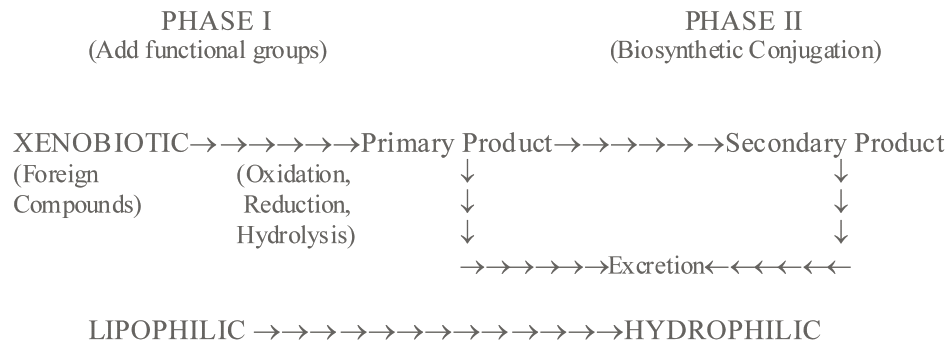


Figure 1. Phase I and Phase II Pathways. Figure adapted from Timbrell, J.A., 1993 Biotransformation of Xenobiotics. General and Applied Toxicology Vol 1, (B. Ballantyne, T. Marrs, and P. Turner Eds.) Macmillan Publishing Co. New York, pp 89 – 116.

Phase I reactions include hydrolysis, reductions, and oxidations of foreign compounds. During Phase I reactions, a functional group is added, making the compound slightly more water soluble and reactive. Phase II reactions which include glucouronidations, sulfonations, acetylations and methylations conjugate the phase I metabolite, usually resulting in a large increase in water solubility and excretability by the compound. The phase I metabolite is not stable, and can become reactive and lead to possible DNA damage. Instead of detoxifying the xenobiotic compound, the opposite can occur with a resulting toxicity increase.

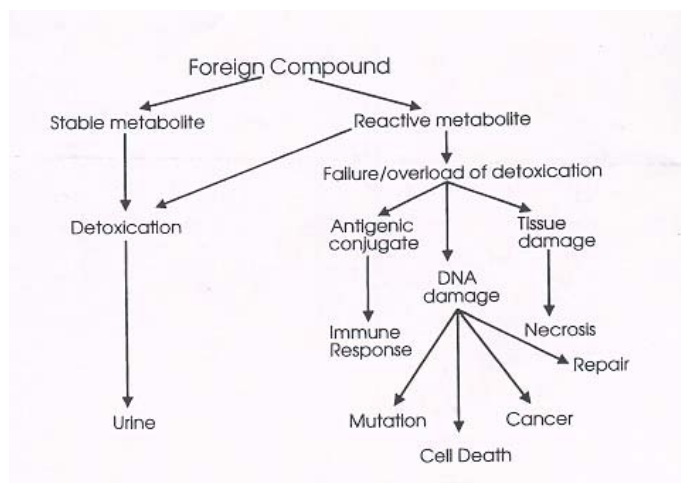


Figure 2. Biotransformation of Xenobiotics. Adapted from Timbrell, J.A., 1993 General and Applied Toxicology Vol 1, (B. Ballantyne, T. Marrs, and P. Turner Eds.) Macmillan Publishing Co. New York, pp 89 – 116.

Genome projects have helped established that CYPs arose from a single ancestral form. Although the molecular species may be different, there are several conserved AA sequences found amongst all CYPs (Omura, 1999). Some prokaryotes and all animals, plants, fungi and eukaryotic organisms have CYPs. CYPs are found in the endoplasmic reticulum and can be species and tissue specific. Their expression can be sex and developmentally regulated. CYPs have been found to be involved in a variety of functions including the biosynthesis and metabolism of steroid hormones and the metabolism of various lipid biofactors such as eicosanoids, vitamin D3 and retinoids (Omura, 1999).

Close to a thousand CYP isoforms have been found. A nomenclature has been established placing the isoforms into families and subfamilies (Omiecinski *et al*, 1999).



CYP - for Cytochrome P450

# - for family (if the same number is used, this indicates greater than 40% protein sequence homology)

Letter – for subfamily (CYPs are in the same subfamily if they have greater than 55% homology in the protein sequence)

# - for gene

For example, the CYP1 family is the family using the aryl hydrocarbon receptor pathway.

There are 2 subfamilies, CYP1A and CYP1B. The CYP1A1 subfamily is made up of 2 genes, CYP1A1 which predominantly uses polycyclic aromatic hydrocarbons as substrates and CYP1A2 which predominantly uses aromatic amines as substrates.

CYP1B1 predominantly uses heterocyclic amines as substrates (Omiecinski *et al*, 1999).

Some CYPs are constitutively expressed and others are only expressed after induction.

Expression varies by species and tissue also.

## **2 - Cytochrome P4501A1 (CYP1A1)**

CYP1A1 is an inducible isoform and is involved in the metabolism of polycyclic aromatic hydrocarbons (PAHs). PAHs are common in combustion products, including cigarette smoke. CYP1A1 has been found in many tissues, including liver, lung, and brain. Expression is usually only detectable after induction and varies between tissues.

Inducers of CYP1A1 are its substrates, the PAHs, which are present in cigarette smoke, charcoaled meat, soot and other common environmental contaminants. Examples include 3-methylcholanthrene (3-MC),  $\beta$ -naphthoflavone (NF), and dioxin (TCDD). Induction can cause an imbalance in detoxification and activation (Whitlock, 1999; Whitlock *et al* 1996).

PAHs induce their own metabolism; they are the substrate of CYP metabolism. They are found almost everywhere in the environment and one can easily be exposed via inhalation, intestinal absorption through eating and drinking, or through skin absorbance. PAHs are then metabolized by CYP1A1, with subsequent involvement of phase II metabolism.

Induction of CYP1A1 by an inducer causes an increase in the transcription of the gene, leading to increased Phase I products, DNA adducts, and somatic mutations, which could then possibly lead to carcinogenesis (Mucci *et al*, 2001).

There are several regulatory proteins involved in CYP1A1 induction. The AhR (aromatic hydrocarbon receptor) and ARNT (aromatic hydrocarbon receptor nuclear translocator) both pre-exist in cells, making induction of CYP1A1 a primary response associated with protein synthesis. Both AhR and ARNT are bHLH/PAS proteins (basic helix loop helix/pers-arnt-sim) that are involved in the regulation and maintenance of basic biological processes, homeostasis, circadian rhythms and development. AhR is

located in the cytoplasm where the inducer can bind to it. Two cytoplasmic factors, HSP90 (heat shock protein 90) and AIP (AhR-interacting protein) enhance receptivity for ligand and help maintain configuration for ligand binding. After ligand binding, both factors dissociate and the liganded AhR is able to enter the nucleus and bind to ARNT. Together, this transcription complex binds to the enhancer element of the CYP1A1 gene. Components interact and the initiation complex forms at the promoter, thus starting transcription and leading to metabolism of the ligand (Whitlock, 1999; Hankinson, 1995).

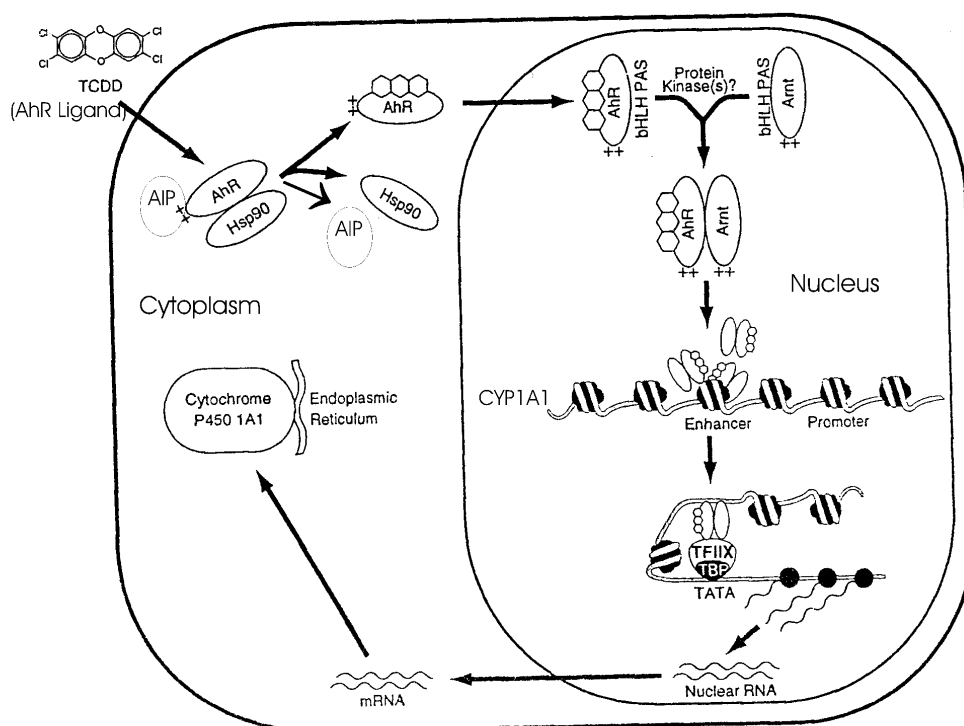


Figure 3. Mechanism of CYP1A1 Induction. Adapted from James Whitlock *et al*, 1996.

### 3 - CYP1A1 and Carcinogenesis

In general, CYP1A1 has low constitutive expression, but induction occurs rapidly and high expression can be induced. CYP1A1 induction can lead to toxicity or carcinogenesis as a result of the oxidation of the PAHs. For example, PAHs are found in cigarette smoke and their oxygenation by CYP1A1 generates arene oxides that can form DNA adducts resulting in lung cancer.

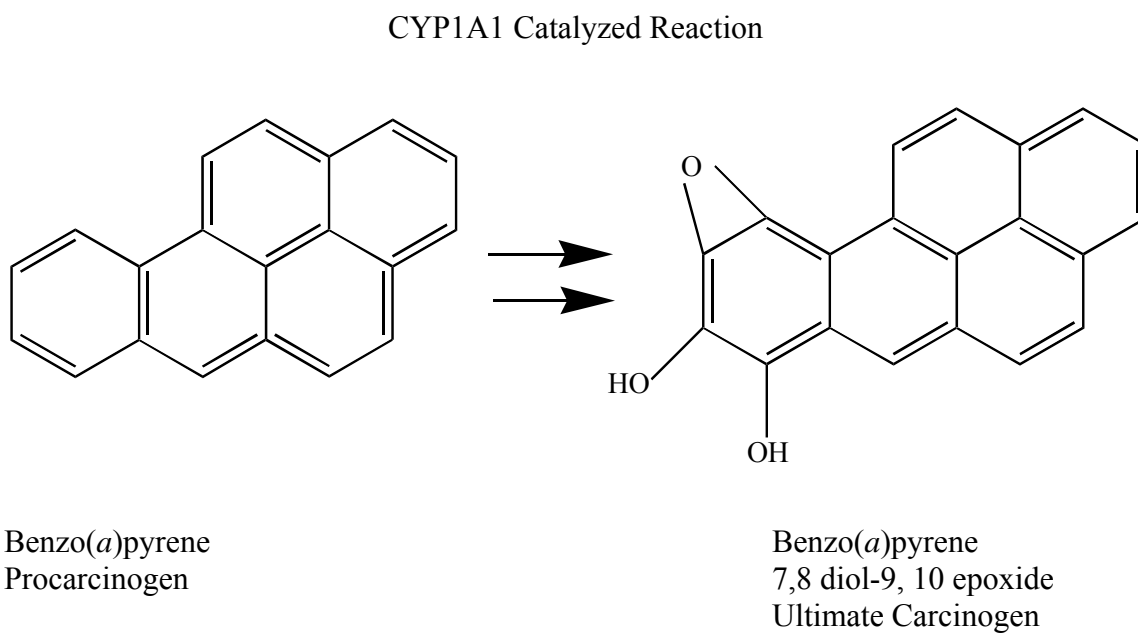


Figure 4. CYP1A1 Reaction: Procarcinogen to Ultimate Carcinogen

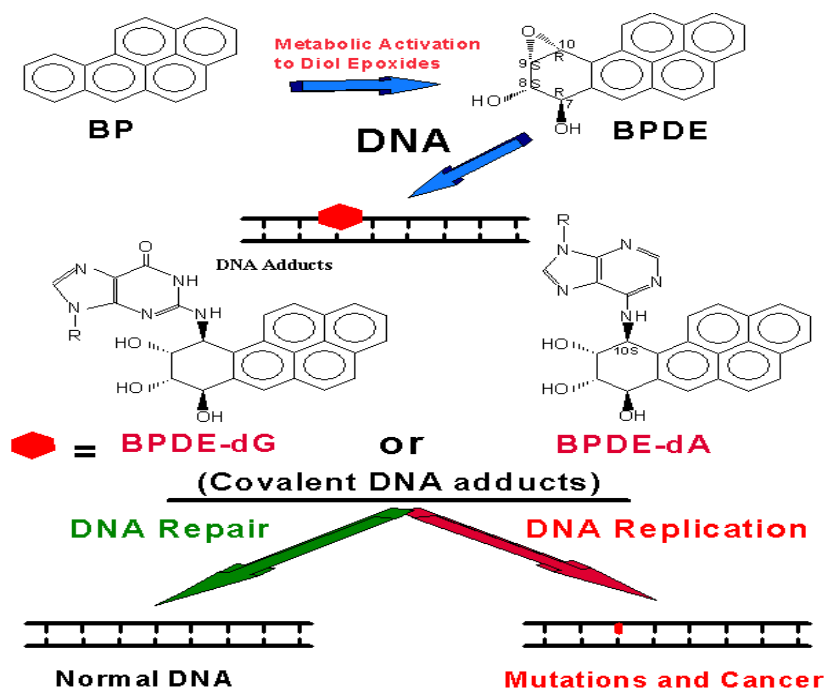


Figure 5. CYP1A1 Metabolism Pathways. Adapted from Geacintov, N.E.; [http://www.nyu.edu/projects/geacintov/images/WEB\\_Figures/WEB\\_PAH3.htm](http://www.nyu.edu/projects/geacintov/images/WEB_Figures/WEB_PAH3.htm)

CYP enzymes have been studied for their role in the activation and detoxification of chemical carcinogenesis. Causes of cancer vary and genetic factors, exposure to chemicals, and diet are all important considerations. Genetics alone is only thought to account for about 5 % of all cancers (Perera, 1997). The rest involved environmental factors which often interact with both genetics and susceptibility (Perera, 1997).

Guengerich and Shimada (1998) summarized evidence of a link between CYP enzymes and cancer:

1 – cancer susceptibility associated with CYP activity.

2- variation in level of CYP enzyme basal expression on the individual exposure patterns (work, diet).

3- variations in drug sensitivity and interactions.

#### **4 - Polymorphisms in CYPs**

Several CYP1A1 polymorphism have been found and there is a wide variety of levels of induced enzyme activity among individuals (Daly *et al*, 1998; Guengerich and Shimada, 1998). In 1973, Kellerman first showed the association between high AHH inductions and bronchiogenic carcinomas in cultured lymphocytes (Smith *et al*, 2001). Recent studies have shown several prominent polymorphs associated with lung cancer. The MspI mutation at the 3' flank that causes a T to C transition and a mutation at the Exon 7 heme binding region that causes an A to G transition which leads to an ILE to VAL substitution are two well known polymorphism in the CYP1A1 gene. A third polymorphism has also been found exclusively among people of African origin – MSP1AA (Smith *et al*, 2001). Most studies on human polymorphisms of CYP1A1 and AhR are still controversial. Some studies have shown increased risk for lung cancer in specific ethnic groups and differences between male and female sex populations for individuals carrying one copy of the variant allele (Smith *et al*, 2001). There is a wide variance in CYP1A1 expression (50X) in human lung tissue, perhaps explaining the

varying outcomes between studies. The variation can be due to both genetic polymorphism or other genes involved in CYP1A1 expression (Alexandrov *et al*, 2002).

Cigarette smoking has long been considered an important factor in lung cancer. Cigarette smoke contains PAHs that are bioactivated by CYP1A1. The link between CYP1A1 polymorphism and lung cancer is still controversial and results are conflicting (Smith *et al*, 2001).

We must also consider the phase II enzymes and their role in carcinogenesis. They are responsible for detoxifying the reactive metabolite that is the product of phase I metabolism. Examples of phase II enzymes include transaminases, acetyltransferases, UDP glucuronyl-, and glutathione-S-transferases (GSTs) that play a huge role in the detoxification of endogenous and exogenous compounds. There are several polymorphisms of GSTs and in most cases, the polymorphs are less effective in the detoxification processes, leading to an increase in cancer risk (Mucci *et al*, 2001).

## 5 - Lung physiology

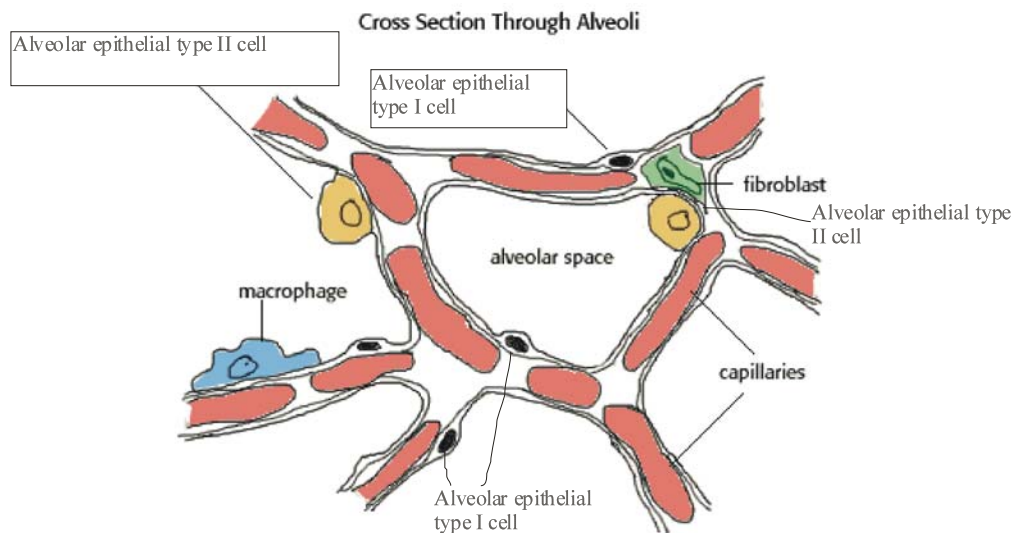


Figure 6. Diagram of lung alveolus. Adapted from [http://www.biology.arizona.edu/chh/problem\\_sets/lung\\_toxicology/graphics/alveolus](http://www.biology.arizona.edu/chh/problem_sets/lung_toxicology/graphics/alveolus).

Over 40 different cell types have been identified in the lung. There are several that are important in our research. Alveolar type I cells facilitate gas exchange, help maintain the alveolar structure, and comprise 96% of the alveolar surface. Alveolar type II cells account for 10 – 15 % of all lung cells but only 4% of the surface area of the alveoli (Castranova *et al*, 1988). On a cell number basis, the type II cells account for 60% of all alveolar cells. Type II cells synthesize and secrete surfactant and are the progenitors of the type I cells. Type II cells also have roles in xenobiotic metabolism and lung damage repair. Once stimulated, they release cytokines and recruit neutrophils. Another epithelial cell, the Clara cell is a non-ciliated airway epithelial cell found mostly



in bronchioles. These cells can differentiate into ciliated cells, participate in xenobiotic metabolism and possibly have a role in surfactant production (Devereux, 1984).

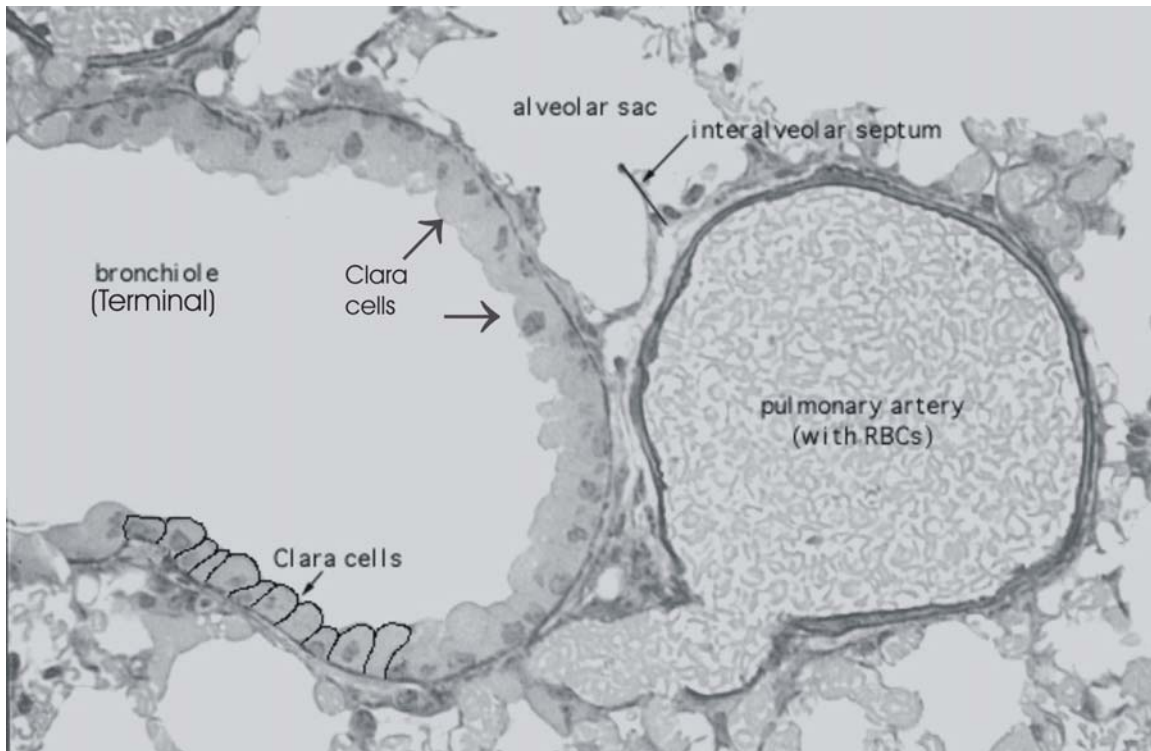


Figure 7. Example of terminal bronchi and Clara Cells. Adapted from <http://www.bu.edu/histology/p/13805loa.htm>.

Another distinct lung cell type, the alveolar macrophage acts as the first line of defense against inhaled particles and bacteria by engulfing the foreign particles. Several studies have been performed showing xenobiotic metabolism in the alveolar Type II cell and the Clara cell (Devereux, 1984). Methods for isolating several cell types have been discovered enabling us to conduct toxicological and mechanistic studies on specific cells (Castranova *et al*, 1988).

Many xenobiotic compounds are aerosolized and easily inhaled by the lungs, making the lungs an easy target organ. In addition, the lung is a highly vascular organ which makes it a potential target organ for circulating xenobiotics. Lung injury can vary depending on physical and chemical nature of compound, size and concentration of particle and the potential clearance of the agent (Thiedemann, *et al*, 1991). The lung has two major clearance mechanisms – ciliated epithelium in the trachea and bronchi and phagocytosis by alveolar macrophages (Stuart, 1976). Particles can become trapped in the lung and the resulting persistence facilitates alterations in lung tissues. Chronic and high exposures to respirable particles can cause overload and lead to fibrosis and cancers (Thiedemann *et al*, 1991; Oberdörster *et al*, 1997). Lung particle overload is associated with lung inflammation, accumulation of particles in centriacinar interstitial and alveolar areas of the lung, type II cell proliferation, and impaired macrophage clearance ability in the rat lung (Oberdörster *et al*, 1995; ILSI Risk Science Institute Workshop, 2000).

## **6 – Respirable Crystalline Silica**

Respirable sized particles reach the alveolar region of the lung. In experimental animal research, there are 2 common ways to expose animals to particles – inhalation or intratracheal instillation. Both procedures have advantages and disadvantages. Intratracheal instillation is inexpensive and a known amount of material is dosed to the lung. However, this method does not accurately reflect human exposures to particles.

Research has shown differences in particle distribution, clearance and injury pattern. The particle distribution is less homogenous in intratracheal instillations than in inhalation exposures, creating a variance of particle burden in the lung (Osier and Oberdörster, 1997). It has been reported that at low level particle exposures the total lung burden is not different between the techniques (Ferin and Feldstein, 1978). A comparison study determined that either method was useful to assess pulmonary responses (Henderson *et al*, 1995). Inhalation can be very expensive and the amount of dosed material is not measured as accurately, but this is the more natural exposure method. (Brain *et al*, 1976; Osier and Oberdörster, 1997; Driscoll *et al*, 2000).

Crystalline silica has many polymorphic forms –  $\alpha$ -quartz,  $\beta$ -quartz, cristoballite (NIOSH Hazard Review, 2002). Individuals can be exposed to crystalline silica in several occupational settings (e.g. coal mining, sand blasting) if protective measures are not taken. Silicosis is chronic lung disease caused by inhalation exposure to respirable crystalline silica. Silicosis can be acute (short exposure to a high concentration) or chronic (long exposure to low concentration). It can also be latent, occurring years after the initial exposure to silica. In 1997, IARC found enough evidence to add respirable crystalline silica to its list of agents that are carcinogenic to humans (IARC Monograph, 1997).

Respirable exposure to crystalline silica causes an inflammatory response in pulmonary cells and tissues (Castranova, 2000; Porter *et al*, 2001; Driscoll *et al*, 1990; Driscoll *et al*, 1991). Several cell types are involved in the development of silicosis. Macrophages try to engulf the particles and phagocytose them (Miller *et al* 1986; Miller *et al* 1987; Miller and Hook, 1990). Lavage studies have shown increases (2x control) in the number of polymorphonuclear leukocytes (PMN's) in silica exposed rats, indicating inflammation. Surfactant production is increased in the alveolar type II cell and they become hyperplastic and hypertrophied in response to silica (Miller *et al*, 1987). Total protein and phospholipid numbers in bronchial lavage fluid also increase in the silica exposed lung (Dethloff *et al*, 1986; Miles *et al*, 1993; Miles *et al*, 1994). The type I cell is damaged and stimulates proliferation of the type II cell to replace the dead type I cell (hyperplasia). Surfactant production increases; the type II cells of silicosis frequently contain abundant surfactant apoprotein and are enlarged (hypertrophic) (Miller and Hook, 1990).

Reactive oxygen species (ROS) are also generated during chronic silica exposures (Vallyathan *et al*, 1998). ROS are produced at both the particle surface and by the phagocytizing cells engulfing the particle (Fubini and Hubbard, 2003). ROS cause cytotoxic lung injury through release of oxidants. These oxidants activate cell signaling pathways, enhancing inflammatory cytokine expression, and activating transcription

factors. Oxidant effects include NF $\kappa$ B activation, AP-1 activation, and TNF alpha production. Apoptosis can also be induced by ROS (Fubini *et al*, 2003).

Oxidants can activate oncogenes or inactivate tumor suppressor genes and lead to carcinogenesis. Hydroxy radicals ( $\cdot$ OH) have been shown to cause DNA breaks and damage that can lead to carcinogenesis via point mutations and deletions (Jackson, 1994).

The release of inflammatory cytokines such as TNF $\alpha$  and IL1 $\beta$ , and IL6 has been shown to inhibit CYP1A1 expression (Jackson, 1994; Morgan, 2001). The mechanism of this down regulation is still being investigated. Several findings include:

The release of TNF alpha can induce the release of NO, which is then able to bind to the heme portion of the CYP. This binding then inhibits activity of the enzyme. NO can also react with oxygen causing a loss in catalytic activity (Paton and Renton, 1998).

NF $\kappa$ B is also induced by TNF $\alpha$ . NF $\kappa$ B plays an important role in several necessary biological processes, including - immune response, inflammatory regulation, cell proliferation, apoptosis, and developmental processes (Ke *et al*, 2001).

NF $\kappa$ B is a heterodimer made up of p65 (relA) and p50. A link between the AhR and NF $\kappa$ B signaling pathway indicates a mechanism for inflammatory mediators to inhibit CYP1A1 expression (Ke *et al*, 2001). RelA (P65 subunit of NF $\kappa$ B) and AhR complexes interact and RelA is able to inhibit activation of CYP1A1 transcription (Tian *et al*, 1999).

Ke *et al*, (2001) suggests that the activation of NF $\kappa$ B remodels the chromatin of the

*CYP1A1* promoter. Their data suggest a possible mechanism for the suppression of *CYP1A1* by inflammation – pro-inflammatory cytokines such as  $\text{TNF}\alpha$  induce NF $\kappa$ B, the RelA subunit interacts with AhR and inhibits activation resulting in down regulation of *CYP1A1* expression (Ke *et al* 2001; Tian *et al*, 1999; Tian *et al*, 2002).

## Chapter 3

### Methods and Materials

#### **1 - Preparation of Silica**

Crystalline Silica ( $\alpha$ -quartz) was obtained from Min-U-Sil, (U.S. Silica, Berkeley Springs, WV). It was fractionated to  $< 5 \mu\text{m}$  using a Donaldson particle classifier. By X-ray diffraction (XRD) it was pure crystalline silica (99.8%) and by X-ray spectrometry (particle analysis) it was 98.7% pure. Surface area measurements were done by nitrogen adsorption technique. Mean surface area of  $< 5 \mu\text{m}$  Min-U-Sil is  $4.7 \text{ m}^2/\text{gram}$ . Mass median aerodynamic diameter (MMAD) of silica is  $3.5 \mu\text{m}$ . Prior to instilling, the silica was sterilized by heating overnight in an oven at  $160^\circ\text{C}$ . Sterile silica particles were weighed and then suspended in 0.9 % sodium chloride (Abbot Laboratories, North Chicago, IL) to produce 5, 10, or 20 mg/0.5 ml solutions. Representative samples of the sterilized silica and the saline solution were analyzed for endotoxin. Measurements were determined by the Limulus Amebocyte Lysate assay (BioWhitaker, Walkersville, MD).

#### **2 - Preparation of CYP Inducers**

Corn oil (Mazola, Englewood Cliffs, NJ) was filtered through a  $0.2 \mu\text{m}$  membrane syringe filter. A 100mg/ml solution of Phenobarbital (Sigma Chemical, St. Louis, MO) was prepared with corn oil. 50 mg/ml solution of  $\beta$ -naphthoflavone (Sigma Chemical, St. Louis, MO) was prepared with corn oil. These solutions were mixed in sterile centrifuge tubes, vortexed and then sonicated for at least 20 minutes.

### **3- The Rat Model of Silicosis**

Male, Sprague-Dawley, Hla:(SD)CVF rats (Hilltop Lab Animals, Scottdale, PA) weighing between 125 and 150 grams on arrival were used for these experiments. Rats were housed in shoebox cages supplied with HEPA-filtered laminar flow air with Alphi-Dri Virgin cellulose chips (Sheperd Specialty Papers, Watertown, TN) and hardwood Beta-chips (NEPCO, Warrensburg, NY) for bedding, and provided tap water and autoclaved ProLab 3500 (Purina Mills Incorporated, St. Louis, MO) rodent diet *ad libitum*. Rats were allowed to acclimate to the animal facility for one week.

Prior to exposure, rats were randomly placed into exposure groups using a table of random numbers. Animals were anesthetized with an intraperitoneal injection (IP) of 35/mg/kg Brevital Sodium (Methohexital sodium, Eli Lilly and Co., Indianapolis, IN) and received an intratracheal instillation (IT) of 0.5 ml of the silica suspension (5, 10 or 20 mg crystalline silica) or the vehicle alone (0.9% sodium chloride). To determine if silicosis suppressed the induction of CYP1A1 or the constitutive expression of CYP2B1 in the lung, rats with and without silicosis were exposed to the CYP1A1 inducer,  $\beta$ -naphthoflavone (NF). Some of these rats were exposed to the hepatic-specific CYP2B1 inducer, phenobarbital, because phenobarbital is a CYP2B1 inducer in the liver and has not been investigated in the silicotic lung.

### **4 - Experiment 1; Exposures to Silica (IT) Classical Inducers of CYP1A1 and CYP2B1**

Seventeen rats received intratracheal instillations of 20 mg silica and twenty rats received saline. Rats were sacrificed 14 days post IT exposure. Rats received CYP inducer and/or vehicle via intraperitoneal injection on days 11 and 13 after IT instillation



as outlined in table M1. These treatments resulted in the following groups of rats: control rats, rats with NF induced pulmonary CYP1A1, phenobarbital exposed rats, phenobarbital exposed rats with NF induced CYP1A1, silica exposed rats, silica exposed rats with NF induced CYP1A1, silica and phenobarbital exposed rats, and silica and phenobarbital exposed rats with NF induced CYP1A1.

Table M1: Experimental Design

Group	Control	CYP1A1 induced	Phenobarbital exposed	Phenobarbital exposed & CYP1A1 induced	Silica Exposed	Silica Exposed & CYP1A1 induced	Silica & Phenobarbital exposed	Silica & Phenobarbital exposed & CYP1A1 induced
Intratracheal instillation Day 0	Saline 0.5 ml	Saline 0.5 ml	Saline 0.5 ml	Saline 0.5 ml	Silica 20 mg	Silica 20 mg	Silica 20 mg	Silica 20 mg
Intraperitoneal injection Day 11	Corn Oil	NF <sup>a</sup>	PB <sup>b</sup>	NF <sup>a</sup> & PB <sup>b</sup>	Corn oil	NF <sup>a</sup>	PB <sup>b</sup>	NF <sup>a</sup> & PB <sup>b</sup>
Intraperitoneal injection Day 13	Corn Oil	Corn Oil	PB <sup>b</sup>	PB <sup>b</sup> & Corn oil	Corn Oil	Corn Oil	PB <sup>b</sup>	PB <sup>b</sup> & Corn Oil
# of animals <sup>c</sup>	5	5	4	5	4	3	4	4

- NF =  $\beta$ -naphthoflavone (50 mg/kg) in corn oil;
- PB = Phenobarbital(100mg/kg)
- Number of animals remaining at time of sacrifice. Experiment started with 5 animals per group. Animal deaths resulted from intraperitoneal injections.

End Points Measured: Pulmonary CYP1A1 and total CYP activity were measured with EROD and ECOD activity, respectively. CYP1A1 and CYP2B1 lung tissue expression was localized with enzymatic immunohistochemistry to the sites of expression. Histopathology was performed to assess lung injury.

### **5 - Experiment 2: Exposures to Silica and BNF, a Classical Inducer of CYP1A1**

12 rats were intratracheally instilled with 20 mg silica and another 12 rats instilled with saline. 11 days after instillation, 6 rats from each group were weighed and injected intraperitoneally with 50mg/kg  $\beta$ -naphthoflavone or vehicle alone (corn oil).

Table M2: Experimental Design for Experiment 2

Group	Control	NF exposed	Silica exposed	Silica & NF exposed
Intratracheal Instillation	Saline	Saline	Silica	Silica
CYP1A1 Inducer	Corn Oil	NF	Corn Oil	NF
# of animals	6	6	6	6

NF =  $\beta$ -naphthaflavone in corn oil

End Points Measured: CYP1A1 and CYP2B1 activity were measured by EROD and PROD activity respectively. CYP1A1 protein was measured by western blot, and CYP1A1 expression was localized within lung tissue by CYP1A1 immunofluorescence using dual immunofluorescence with cytokeratin 8, a marker of alveolar type II cells in the lung alveolus. Histopathology was used to assess lung injury.

### **6 - Experiment 3: Silica Dose Response Study with Exposure to Classical Inducer of CYP1A1**

Five rats were intratracheally instilled with saline, 5 mg silica, or 10 mg silica and four rats were intratracheally instilled with 20 mg silica. Eleven days later, all rats received a 50 mg/kg IP injections of NF.

Table M3: Experimental Design for Experiment 3

Groups	NF Control	NF/low dose	NF/mid dose	NF/high dose
Intratracheal Instillation	Saline	5 mg Silca	10 mg Silica	20 mg Silica
CYP1A1 Inducer	NF	NF	NF	NF
# of animals*	5	5	5	4

NF =  $\beta$ -naphthaflavone in corn oil

\* = Number of animals at time of sacrifice. One animal died as a result of intraperitoneal injection.

End Points Measured: CYP1A1 and CYP2B1 activity were measured by EROD and PROD activity, respectively. CYP1A1 and CYP2B1 protein were measured by western blot. Histopathology was used to assess lung injury.

### **7 - Tissue Collection (Experiments 1, 2, and 3)**

On day 14 after intratracheal instillation, the rats were euthanized. The rats were euthanized by an intraperitoneal injection of an overdose of sodium pentobarbital followed by exsanguination via transection of the abdominal aorta. The lungs were removed and the right lung lobes were isolated, tied off and removed. The right lung lobes were placed on ice and microsomes were isolated as subsequently described for biochemical analysis. The left lung lobe was inflated via the trachea with 3 ml of neutral buffered formalin, tied off and placed in a sealed bag with 10% neutral buffered formalin (10% NBF). The tracheobronchial lymph node, parathymic lymph node, liver and kidney were also collected and placed in 10% neutral buffered formalin.

## **8 - Biochemical Analysis; Preparation of Lung Microsomes (Experiments 1, 2, and 3)**

Microsomes were prepared as described by Miles *et al*, 1993 and Ma *et al*, 2002. At necropsy, the right lung lobes were weighed and placed on ice. The lung tissue was chopped using a McIllwain tissue chopper (The Mickle Engineering Co., Gomshall, Surrey, UK). Tissues were chopped atleast 4 times with slice thickness set at 5 mm. Ice cold buffered solution (145mM KCl, 30mM Tris-HCl, 1.9mM KH<sub>2</sub>PO<sub>4</sub>, and 3mM MgCl<sub>2</sub>, pH 7.4) was added to the tissue homogenate at 25% wt/vol ratio. A teflon-glass Potter-Elvehjem homogenizer was used to make a homogenate (16 complete passes with the pestle). Four sequential centrifugations were performed to obtain the microsomal fraction. The first spin was for 10 minutes at 1000 times g. The next three centrifugations were for 20 minutes each at 3,000, 10,000, and 15,000 times g. Using an ultracentrifuge, the microsomal fraction was obtained by centrifuging at 105,000 x g for 75 minutes. The microsomal pellet was resuspended in cold buffered solution, aliquoted and frozen at -80°C.

## **9 - Biochemical Analysis; Measurement of CYP1A1 protein activity by Ethoxyresorufin-O-deethylase (EROD) Activity (Experiment 1, 2, and 3)**

CYP1A1 protein activity was measured in isolated microsomes stored at -80C. EROD was measured as previously described (Burke and Mayer, 1974; Burke *et al*, 1985; Miles *et al*, 1993). Reactions were carried out in fluorimeter cuvettes that contained 150 µl of microsomes, 2.78 ml phosphate buffer (145 mM KCL, 1.9 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM K<sub>2</sub>HPO<sub>4</sub>, 30 mM Tris-HCL, and 3 mM Mg Cl<sub>2</sub>; pH 7.6), 9 µl 7-ethoxyresorufin (3 µM), 7.5 µl dicumarol (25µM). The dicumarol was added to prevent the

interference resulting from the elevated quinone oxidoreductase levels found in preparations from animals pretreated with xenobiotics (Burke *et al*, 1985). The mixture was allowed to equilibrate for 1 minute at 37°C and then 50 µl of reduced form of NADPH (28.8 mM) was added to initiate the reaction. The reaction for the first experiment was carried out for 8 minutes and was measured at various time points to replicate the method of Miles and co-workers (Miles *et al*, 1993). For the second and third experiments, the reaction was measured by measuring the optical density every minute for 10 sequential minutes (Ma *et al*, 2002). Under these conditions, increases in the fluorescence are due solely to CYP dependent formation of a single metabolite, resorufin, from the substrate used (Burke *et al*, 1985). The fluorimetric settings were 530 nm and 585 nm for the excitation and emission respectively, and 6.5 nm for the excitation and emission slit. A standard curve was determined after each run using a 10 µM resorufin solution prepared from 2.35 µg resorufin in 1 ml DMSO. For compatibility with the previous experiments of Miles and co-workers (Miles *et al* 1993; Miles *et al*, 1994) EROD activity for the first experiment was expressed as pmol/mg/microsomal protein/8 minutes or as pmol/lung/8 minutes. For the second and third experiments, EROD was expressed as pmol/min/mg microsomal protein or pmol/min/lung for comparability with other studies of particle effects in the lung (Ghanem *et al*, 2004).

### **10 - Measurement of Cytochrome P450 – ECOD (Experiment 1)**

ECOD was measured as previously described (Ullrich and Weber, 1972; Burke *et al*, 1985; Miles *et al*, 1993). As with EROD, the activity of ECOD was measured using microsomes stored at -80°C. The cuvettes contained a total volume of 6 ml, which

contained of 0.1mg protein per milliliter incubation medium lung microsomes, 0.5mg/ml bovine serum albumin, 0.48 mM NADPH, and 0.5 mM of 7-Ethoxycoumarin -*O*-deethylase. The reaction was initiated by the addition of 7- Ethoxycoumarin -*O*-deethylase in dimethyl sulfoxide (DMSO) (5 $\mu$ l/ml) and carried out at 37 °C for 30 minutes. Umbelliferone, the reaction product, was measured after 30 minutes using a fluorometer as described by Ullrich and Weber, 1972. A model LS-50 Luminescence Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) was used for measurements and results were expressed as pmol/mg/microsomal protein/30 minutes or pmol/lung/30 minutes.

### **11 - Measurement of the CYP2B1 - Dependent Enzymatic Activities**

#### **(PROD) (Experiments 2 and 3)**

This assay was performed as previously described (Burke *et al*, 1985; Ma, et al, 2002). The procedure was similar to EROD except the sample volume of the microsomes used was increased (300  $\mu$ l). Also the substrate used for the reaction was 7-pentoxy-resorufin. Resorufin was also used to determine the standard curve. Results were expressed as pmol/min/mg/microsomal protein or pmol/min/lung.

### **12 - Determination of the Total Lung Proteins: (Experiments 1, 2, and 3)**

The bicinchonic acid (BCA) method (Pierce, Rockford, IL) was used to measure the protein content of the lung microsomes. Bovine serum albumin was used as the standard and the 96 well plates were read on a spectrophotometer (Spectramax 250, Molecular Devices Corporation, Sunnyvale, CA).

**13 - Western Blot Analysis – (Experiment 2 and 3:CYP1A1, Experiment 3: CYP2B1)**

Lung microsomes were used to quantify the amount of CYP1A1 or CYP2B1 per mg microsomal protein. The procedure was performed as described by Ma *et al*, 2003. Microsome samples were thawed on ice and protein was placed in an eppendorf tube (75 µg for Experiment 2 CYP1A1, 37.5 µg for Experiment 3 CYP1A1, and 20 µg for Experiment 3 CYP2B1). BNF treated rat liver microsomes (Xenotech, Kansas City, Kansas) diluted 1:100 with 2X loading buffer (Invitrogen, Carlsbad, CA) were used as a positive control for CYP1A1 expression and phenobarbital treated rat liver microsomes (Xenotech, Kansas City, Kansas) diluted 1:10 with 2X loading buffer were used as a positive control for CYP2B1. The rainbow color marker (Multimark™, Invitrogen, Carlsbad, CA), and the molecular weight marker (Magicmark™, Invitrogen, Carlsbad, CA) were mixed 1:2 with 2X SDS Loading buffer (Invitrogen, Carlsbad, CA). All samples were then vortexed, heated at 95 °C for 5 minutes and allowed to cool before vortexing again and centrifuged for 10 – 15 seconds. 1.5 mm tris-glycine gels (Invitrogen, Carlsbad, CA) were used to load the samples. Microsome samples were loaded into the gel. 10 µl of positive control and color marker, and 5 µl of molecular weight marker were also loaded on every gel. Gels were electrophoresed in 1X running buffer (Invitrogen, Carlsbad, CA) using an Xcell SureLock Mini Cell™ (Invitrogen, Carlsbad, CA) with a BIORAD power supply (BioRad, Hercules, CA). After 90 minutes at 125 volts, the gels were removed and transferred onto nitrocellulose membranes using the Xcell II™ Blot Module (Invitrogen, Carlsbad, CA). The transfer was completed after 90 minutes at 25 volts in transfer buffer. Gels were then discarded and the membranes

were blocked with a 5% milk solution for at least one hour on a rocker. The membranes were then washed in tris-buffered saline with 0.1% Tween (TBS-T) for 5 to 15 minutes. The primary antibody, a polyclonal, affinity purified rabbit anti-rat CYP1A1 (Xenotech, Kansas City, Kansas) or Mouse anti-rat CYP2B1 (Xenotech, Kansas City, Kansas) was diluted 1:1000 with 1% milk and allowed to incubate on the membrane overnight on a rocker at 4 C. The membrane was then washed in TBS-T for 5 to 15 minutes each before applying the secondary antibody. The secondary antibody was either HRP conjugated goat anti-rabbit IgG (for CYP1A1) or goat anti-mouse IgG (for CYP2B1) (SantaCruz Biotechnology, Santa Cruz, CA) and was diluted 1:2000 in 1% milk. Incubation was at room temperature for one hour on a rocker. Membranes were washed again and detection was performed using chemiluminescent reagent (ECL2109, Amersham Buckinghamshire, England). The membranes were incubated for 1 minute with the ECL reagents, blotted, covered with plastic and exposed to undeveloped film for 30 to 60 seconds. Film was developed using an automatic developer (Kodak, Rochester, NY). Film images were scanned using the Eagle Eye II (Stratagene, La Jolla, CA) and the scanned images were quantified using commercial morphometry software (Image Quant 5.1 Molecular Dynamics/Amersham Biosciences, Piscataway, NJ). The volume of each band was quantified 3 separate times and averaged. Data were compared as percent positive control. Negative numbers were considered as zeros.



#### **14 - Histologic Processing of Tissues (Experiments 1, 2, and 3)**

A section of the left lung lobe was processed and embedded in paraffin within 24 hours of necropsy. The remaining tissues were stored in 10% NBF until processing. Slides stained with Hematoxylin and Eosin (H & E) and Masson's Trichrome were prepared from the left lung. Additional tissue sections of each left lung lobe block were cut at 5 microns and placed on silane treated slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA) for immunostaining.

#### **15 - Hematoxylin and Eosin Staining of tissues (Experiments 1, 2, and 3)**

The slides were stained on the Shandon Lipshaw Linistain GLX (Thermo Shandon, Pittsburgh, PA) slide stainer. The stainer is set up as follows: 5 baths of xylene, 100% alcohol, 95% alcohol, distilled water, Harris hematoxylin, distilled water, bluing solution, distilled water, 95% alcohol, eosin 95% alcohol and 100% alcohol. The slides were placed on the slide stainer and heated at 65° C for several minutes before being placed in the first bath. Each bath is approximately 30 seconds long. At the end, the slides were incubated in xylene and coverslipped with Permount.

#### **16 - Masson's Trichrome Staining of tissues (Experiments 1, 2, and 3)**

The slides were deparaffinized as follows: 3 baths of xylene for 6 minutes each, and a 3 minute bath in each of the following: 100% alcohol, 95% alcohol, and 80% alcohol. The slides were finally placed in distilled water for at least 5 minutes before beginning staining. Staining was performed as described by Masson, (1929). Using glass staining dishes, slides were placed in Bouin's solution for 1 hour at 56°C then rinsed in running water until yellow color disappears. Slides were rinsed in distilled water and placed in Weigert's iron hematoxylin for 10 minutes and then rinsed in running water for

10 minutes. After another rinse in distilled water, slides were placed in Biebrich scarlet-acid fuchsin solution for 2 minutes and rinsed in distilled water. Slides were then placed in Phosphomolybdic-phosphotungstic acid solution for 10 to 15 minutes, followed by a 5 minute bath in Aniline blue solution. The slides were rinsed in distilled water, placed in glacial acetic acid solution for 3 to 5 minutes, and dehydrated and mounted with permount.

### **17 - Histopathology: Experiments 1, 2, and 3**

Tissue sections from all experiments were examined by a board certified veterinary pathologist who was blinded to exposure status. Tissue sections from the third experiment were scored semi-quantitatively for statistical analysis as previously described (Hubbs *et al*, 1997). The severity and distribution of the morphologic changes were recorded and converted to quantitative scores for each slide. The numerical transformation is given in Table M4. The pathology score is the sum of the severity and distribution scores which produces a score with a potential range from 0 – 10.

Table M4

<b><u>Score</u></b>	<b><u>Severity</u></b>	<b><u>Distribution</u></b>
0	None	None
1	Minimal	Focal
2	Mild	Locally extensive
3	Moderate	Multifocal
4	Marked	Multifocal and Coalescent
5	Severe	Diffuse

## **18 - Enzymatic Immunohistochemistry for CYP1A1 (Experiment 1)**

A modification of the labelled streptavidin-biotin (LSAB) procedure provided by the manufacturer in the commercial kit (LSAB2 Kit, DAKO, Carpinteria, CA) was used in these studies. In this procedure, slides are deparaffinized, antigenicity is retrieved, non-specific peroxidase activity and binding is blocked, a primary antibody is applied, followed by a biotinylated secondary antibody and a streptavidin-enzyme complement. A substrate chromogen is applied to visualize the color reaction and location of the antibody.

For each animal, a slide was incubated with rabbit anti-CYP1A1 polyclonal antibody or rabbit control serum (negative control). Because  $\beta$ -naphthaflavone (NF) is a strong inducer of CYP1A1, a slide from an animal treated with IT saline and IP NF was included with every group of slides stained to serve as a positive control.

**Deparaffinizing:** The slides were heated in an oven at 65° C for 20 minutes and deparaffinized in fresh solutions of xylene and alcohols. Deparaffinization consisted of six 30 second baths of xylene, one 30 second bath of 100% alcohol and a 30 second bath of 95% alcohol, and a 5 minute bath in distilled water.

**Blocking for non-specific peroxidase activity and binding:** After deparaffinizing, slides are placed in a humidity chamber and 3% hydrogen peroxide solution was dropped on the slides for a 5 minute incubation at room temperature. Slides were rinsed with distilled water and Tissue Conditioner (Biomedex, Foster City, CA) was applied for 5 minutes at 37° C. Slides were blotted and placed in a coplin jar with Tris Buffered Saline (TBS (Dako, Carpinteria, CA) for 5 minutes at room temperature.

Primary antibody application: The primary antibody, rabbit anti-rat CYP1A1/1A2 (Amersham, Buckinghamshire, England) was reconstituted by adding 300 microliters of 1% BSA/PBS and then diluted 1:4 with Antibody Diluent (Dako, Carpinteria, CA). Rabbit control serum (Biogenex, San Ramon, CA) was applied in place of the primary antibody on negative control slides. The primary antibody or control rabbit serum were applied and the slides incubated overnight at room temperature in the humidity chamber.

Secondary antibody, label antibody and chromogen application: The following morning the slides were rinsed with distilled water and placed in TBS for 5 minutes. The secondary antibody, biotinylated anti-rabbit immunoglobulins (LSAB2 KIT, Dako, Carpinteria, CA) was dropped on the slides, and the slides were incubated for thirty minutes at room temperature in the humidity chamber. The slides were rinsed with distilled water and placed in TBS for 5 minutes at room temperature. For labeling, streptavidin peroxidase (LSAB2 Kit, Dako, Carpinteria, CA) was dropped on the slides for thirty minutes at room temperature. After rinsing in distilled water and incubating for 5 minutes in TBS, the chromogen, 3-Amino-9-ethylcarbazole (AEC) (Dako, Carpinteria, CA) was applied for 15 minutes at room temperature. These procedures were done according to the directions from the manufacturer, Dako (Carpinteria, CA).

Counterstaining and coverslipping: The slides were rinsed in distilled water and counterstained with Aqueous Hematoxylin (Biomedex, Foster City, CA) for 1 – 2 minutes. The slides were rinsed with tap water and covered with Crystal Mount (Biomedex, Foster City, CA) mounting media. The slides were allowed to completely dry before coverslipping with a coverglass and Permount (Fisher Scientific, Pittsburgh, PA).

Slide Visualization and Morphology: Using an image analysis system (Image-1, Universal Imaging, West Chester, PA) the slides were morphologically quantified. From 8 to 10 small airways per slide, the number of positive CYP1A1 staining Clara cells per mm basement membrane were counted and recorded. Ten different frames of alveolar regions measuring  $0.035 \text{ mm}^2$  each were examined and the number of CYP1A1 positive plump epithelial cells were counted and recorded.

### **19 - Enzymatic Immunohistochemistry for CYP2B1 (Experiment 1)**

The same procedure as previously described for CYP1A1 was used for CYP2B1 immunohistochemistry except for the use of a different primary antibody with a different dilution and incubation time. The primary antibody (rabbit anti-rat CYP2B1, Amersham, Buckinghamshire, England) was reconstituted with  $300 \mu\text{l}$  of 1% BSA/PBS. The primary antibody was then diluted 1:10 with diluent (Dako, Carpinteria, CA) and dropped on the slides. The slides were incubated overnight at  $4^\circ\text{C}$  in the humidity chamber. Non-immune rabbit serum was applied to selected slides as a negative control. Image analysis was also performed as described above.

### **20 - Immunofluorescence Staining for CYP1A1/Cytokeratin 8 (Experiment 2)**

Dual immunofluorescence for CYP1A1 and cytokeratin 8 was performed on the lung tissue to further define the location of CYP1A1 in the alveolus during silicosis and xenobiotic (BNF) exposure. Cytokeratin 8, is an intermediate filament which is part of the cytoskeleton in alveolar epithelium of the rat (Schlage *et al*, 1998). Cytokeratin 8 expression is generally weak in alveolar type I cells when compared to alveolar type II cell expression and was used to identify type II cells within the pulmonary alveolus.

**Preparation of slides for reacting with primary antibodies:** Slides were heated in an oven at 65°C for 20 minutes prior to deparaffinization. For deparaffinizing, slides were placed in 3 separate xylene baths for 6 minutes each, followed by 3 minutes in each of the following: 100% alcohol, 95 % alcohol, and 80% alcohol. These were followed by a 5 minute bath in distilled water. To increase antigenicity, we modified a method of microwave antigen retrieval (Shi *et al*, 1991). A 0.01M EDTA solution (pH 8.0), was prepared and used for microwave antigen retrieval. The slides were placed in a microwave safe plastic staining rack and container that was filled with the EDTA solution. The container was placed in the microwave (Kenmore # 89626, Sears Roebuck and Co, Chicago, IL) and heated for 1 minute 45 seconds on high to bring the solution to just under 100° C. The defrost cycle on the microwave was set for 6 minutes to keep the solution at a temperature just under boiling. After 6 minutes, additional EDTA solution was added to fill the container and the defrost cycle was repeated for 6 minutes. After microwaving, the solution was removed from the microwave and the slides remained in the hot solution for 20 minutes. The slides were then rinsed in distilled water and placed in a coplin jar with distilled water for 5 minutes. Unless otherwise specified, the remaining steps were performed in a humidity chamber to prevent the slides from drying. A 5% solution of IgG-free BSA (Sigma Chemicals, St. Louis, MO) in PBS was applied to the slides as a block for 10 minutes at room temperature. The slides were rinsed with distilled water and 5% pig serum in PBS (Biomedex, Foster City, CA) was applied for 10 minutes at room temperature.

#### Reaction of lung tissue with primary antibodies:

The first primary antibody, sheep anti-human cytokeratin 8 (The Binding Site, Birmingham, United Kingdom, England) was diluted 1:10 with Dako Diluent. The second primary antibody, rabbit anti-rat CYP1A1 (Xenotech, Kansas City, KS) was diluted 1:5 with the 1:10 diluted cytokeratin 8 to produce a suspension containing a 1:5 CYP1A1 and 1:10 cytokeratin 8 antibody solutions. Using capillary action, the suspension containing both primary antibodies was applied to the slides. The slides were incubated overnight at room temperature then placed in an oven at 37°C the following morning for 2 hours. The slides were then rinsed thoroughly with distilled water. Rinsing the slides consisted of a 5 minute bath in a coplin jar with distilled water repeated 3 times.

The secondary antibodies, Alexa 594 goat anti-rabbit IgG super highly cross absorbed, and Alexa 488 donkey anti-sheep IgG, (Molecular Probes, Eugene, OR) were diluted 1: 20 with PBS, vortexed, and centrifuged for 5 minutes. The supernatant from each secondary was applied to each slide for 2 hours at room temperature and kept in a dark environment. The slides were then rinsed very well with distilled water as described previously and coverslipped with a glass coverslip and GelMount (Biomedex, Foster City, CA). The slides were stored in the dark and refrigerated until analyzed.

#### Visualizing the sites of CYP1A1 and Cytokeratin 8 expression:

Using a Quantix Digital Camera (Photometrics, Tucson, AZ) and an Olympus AX70 photomicroscope (Olympus, Melville, NY), images were captured as bitmap files. CYP1A1 expression was visualized as red fluorescence using a Texas Red cube with an excitation at 460 – 500 nm and bandpass emission of 510 - 560 nm (U-M41004 HQ-TR,

Olympus, Melville, NY). Cytokeratin 8 expression was visualized as green fluorescence using a band pass FITC cube with an excitation at 432.5 - 487.5 nm and bandpass emission of 607.5 - 682.5 nm (41001 HQ-FITC, Olympus, Melville, NY). Five images each of both proximal alveolar regions near terminal bronchi, and random, alveolar regions between visible terminal bronchi were captured as 1024 x 1024 pixel arrays and saved as 12.2 MB bitmap files. Each image was taken using the green cube, red cube, and combined image.

## **21 - Quantification of CYP1A1 and Cytokeratin 8 IF staining in Alveoli**

### **(Experiment 2)**

Each slide had two regions of interest – the proximal alveolar region (area adjacent to the terminal bronchioles, Figure 1A) and the random alveolar region (area between visible terminal bronchioles, Figure 1B). Photos with vascular endothelium from large vessels were avoided for both regions. From each slide, 5 images (14,527.68 micrometers<sup>2</sup>) were captured from each region using an Olympus AX70 Microscope (Melville, NY) with fluorescence capabilities, a 60 x objective, and a digital camera (Quantix, Photometrix, Tuscon, AZ). The settings for brightness, contrast, and gamma were held constant for each image.

Every image was captured and saved on a CD as red, green, and combined red and green image. Each image was then analyzed by commercial morphometry equipment (Metamorph, Universal Imaging, West Chester, PA). The average from each of the 5 images was calculated for each rat.



The following measurements were made on each region:

- 1) Percent of the region that fluoresced red
- 2) Area within the region that fluoresced red
- 3) Percent of the region that fluoresced green
- 4) Area within the region that fluoresced green, and
- 5) Area of the region that fluoresced both red and green

Red fluorescence indicated sites of CYP1A1 expression. Green fluorescence indicated sites of cytokeratin 8 expression, which was primarily in airway epithelial cells in the airways and alveolar type II cells in alveoli.

The formulas are defined as follows:

The percent CYP1A1 and cytokeratin 8 and the area of CYP1A1 and cytokeratin 8 are values directly measured by Metamorph software.

Area of CYP1A1 expressed in non-cytokeratin 8 areas (non type II cells) = percent of CYP1A1 *not* in Cytokeratin 8 areas (directly measured by Metamorph software) X total area of CYP1A1 in image (measured directly by Metamorph software, in square microns).

Area of CYP1A1 expressed in Cytokeratin 8 areas (alveolar type II cells) = percent of CYP1A1 in Cytokeratin 8 areas (directly measured by Metamorph software) X total area of CYP1A1 in image (measured directly by Metamorph software, in square microns).

Proportional expression of CYP1A1 in alveolar type II cells = percent of CYP1A1 in Cytokeratin 8 areas (directly measured by Metamorph software) X total area of CYP1A1 expression in image (measured directly by Metamorph software, in

square microns) / Total area of cytokeratin 8 expression in image (measured directly by Metamorph, in square microns).

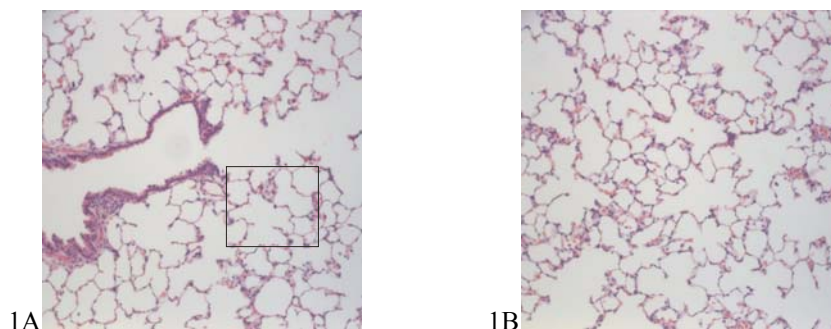


Figure 1 Sampling of pulmonary alveoli by digital image capture.  
 1A . A representative sample digitally captured and analyzed for Proximal Alveolar Regions. Care was taken not to include open airway or any part of the terminal bronchus.  
 1B. The Random Alveolar Regions are areas between and away from visible terminal bronchioles. Care was taken to get areas from separate areas of the slides and between airways.

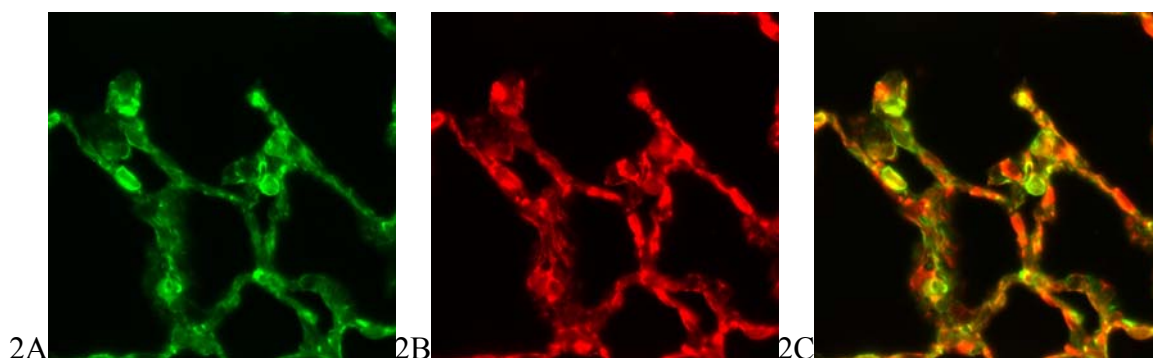


Figure 2 . Example of Double-Immunofluorescence Staining. The bright green staining in A represents Cytokeratin 8 positive cells. The bright red staining represents CYP1A1 positive cells. C is a combined (colocalized) image of cytokeratin 8 and CYP1A1 immunofluorescence. Yellow staining represents areas that are both CYP1A1 and Cytokeratin 8 positive.

## **22 - Statistical Analysis(Experiments 1, 2 and 3)**

All analysis was performed using SAS Version 8.2. Data from the first experiment was analyzed as a Three Way Analysis of Variance (ANOVA) comparing IT exposure, CYP1A1 inducer, and CYP2B1 inducer. Pairwise comparisons were done

using Fishers Least Significant Difference test (LSD). The second experiment was analyzed as a Two Way Anova comparing IT exposure and inducer effects. Pairwise comparison tests were also done using Fishers LSD. A One Way Anova was used to compare the IT exposures in the third experiment. Histopathology scores were analyzed non-parametrically by the Kruskal-Wallis test. For all data, values of  $P < 0.05$  were defined as statistically different.

## Chapter 4

### Results

#### 1 - Histopathology – Light Microscopy findings in silica-exposed rats

During experimental silicosis, rats developed significant accumulations of lipoprotein within alveolar spaces two weeks after instillation. Alveolar type II cells, which produce lipoprotein, were also hypertrophied and hyperplastic. These changes were accompanied by inflammation. Alveolar lipoproteinosis, alveolar type II cell hyperplasia, and alveolar inflammation were seen in all silica exposed rats but were not seen in any saline-instilled control rats.

The morphologic changes observed in the lungs and lymph nodes in the third experiment were converted to quantitative scores and analyzed statistically. The following morphological changes in the lung: alveolitis ( $p = 0.0123$ ), lipoproteinosis ( $p = 0.0008$ ), and type II cell hypertrophy and hyperplasia ( $p = 0.0069$ ), were significantly associated with silica exposure. Inflammation in the tracheobronchial and parathymic lymph nodes was also significantly associated with silica exposure. A dose response relationship was not observed among the silica exposed groups (Table R1).

Table R1 Pathology Scores from Dose Response Experiment (Experiment 3)<sup>1,2</sup>

Group	Lipoproteinosis	Type II Cell Hyperplasia and Hypertrophy	Alveolitis
NF and Saline	0 (0)	0 (0)	0 (0)
NF and 5 mg Silica	5 (4 - 6)	6 (4 - 6)	6 (5 - 7)
NF and 10 mg Silica	5 (4 - 6)	6 (6 - 6)	6 (5 - 7)
NF and 20 mg Silica	5 (5 - 5)	6 (6 - 6)	6 (6 - 7)

1. Pathology score is the sum of the severity (the degree of change) and the distribution (the extent of change) scores.
2. Score is given as median (range)

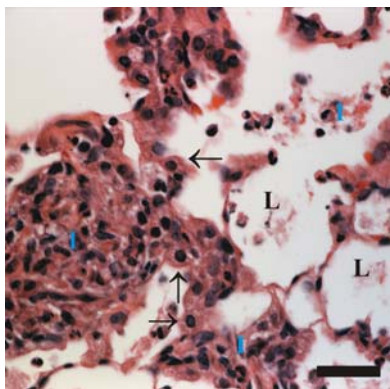


Figure 3 Example of silicotic rat lung. Morphologic changes include lipoproteinosis (L), type II cell hyperplasia (arrows), and inflammation (I) which is manifested as alveolitis. Bar equals 30 microns.

## **2 - Endotoxin Analysis**

Representative samples of the sterilized silica and the saline solution were analyzed for endotoxin and all endotoxin concentrations were found to be under 0.2 ng/ml.

## **3 - Experiment 1 -- Exposures to Classical Inducers of CYP Isoforms: The effect of inducers of cytochrome P450 on pulmonary CYP2B1 immunohistochemistry in silicosis**

Exposing control and silica-exposed rats to the hepatic CYP2B1 inducer, phenobarbital (PB, 100 mg/kg, IP, twice) did not affect the airway and alveolar distribution of CYP2B1. Similarly, exposing control and silica-exposed rats to the pulmonary CYP1A1-inducer,  $\beta$ -naphthoflavone (NF, 50 mg/kg once, IP, once, 3 days prior to necropsy), did not alter the airway and alveolar distribution of CYP2B1. Exposing control and silica-exposed rats to both PB and NF (50 mg/kg NF once, 100 mg/kg PB twice) also did not affect the airway and alveolar distribution of CYP2B1. In particular, the hypertrophied alveolar type II cells of silicosis remained devoid of immunohistochemically detectable CYP2B1. The number of morphometrically

measurable CYP2B1 expressing cells was unaffected by silica, NF, PB, or combinations of these exposures (Figure 4).

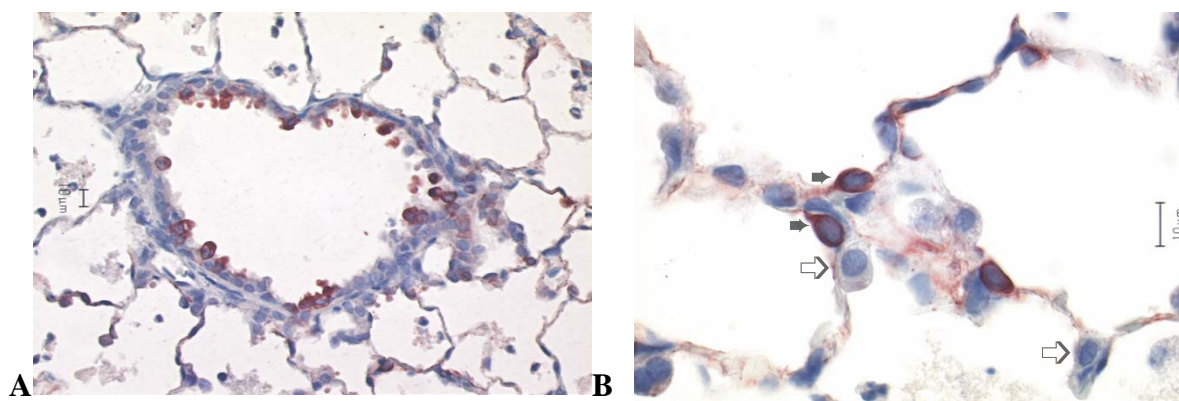


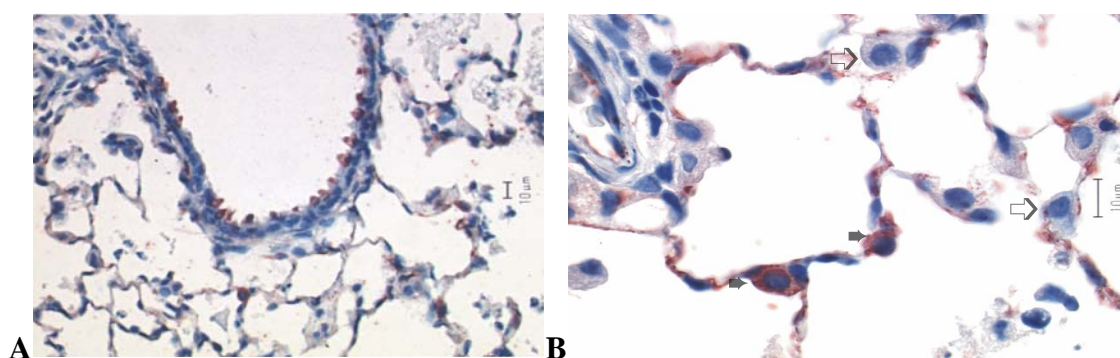
Figure 4. CYP2B1 Immunohistochemistry A. Positive non-ciliated airway epithelial cells were found in all groups. B. Positive cuboidal alveolar epithelial cells (→) were also found in all groups. Cuboidal alveolar cells without immunoreactivity(⇨) were also found in the silica exposed animals.

### **3A - The effect of inducers of cytochrome P450 on pulmonary CYP1A1**

#### **immunohistochemistry in silicosis**

CYP1A1 was consistently detected in the lungs of all rats receiving the CYP1A inducer, NF. No immunohistochemical evidence of CYP1A1 was seen in rats that did not receive NF. NF consistently resulted in the appearance of immunohistochemically detectable CYP1A1 in alveoli and airways. In airways, NF exposure resulted in CYP1A1 expression in nonciliated airway epithelial cells. In the vasculature, NF exposure resulted in CYP1A1 expression in endothelial cells. In the alveolus, the thin cytoplasm of alveolar type I cells appeared to contain immunoreactive CYP1A1, although reactivity of capillary endothelial cells could not be excluded. Some alveolar lining cells with the morphologic appearance of alveolar type II cells contained CYP1A1. However, the hypertrophied alveolar type II cells which are characteristic of silicosis did not express

immunohistochemically detectable CYP1A1. Morphometry indirectly showed that silicosis did not significantly affect the number of airway or cuboidal alveolar epithelial cells (presumptive alveolar type II cells) expressing CYP1A1 after NF exposure (Figure 5). Due to the thin cytoplasm of alveolar type 1 cells and alveolar capillary endothelial cells, the number of these cells expressing CYP1A1 could not be counted by morphometric analysis of immunohistochemically stained sections. PB, an inducer of hepatic CYP2B1, had no effect on pulmonary CYP1A1 expression.



C

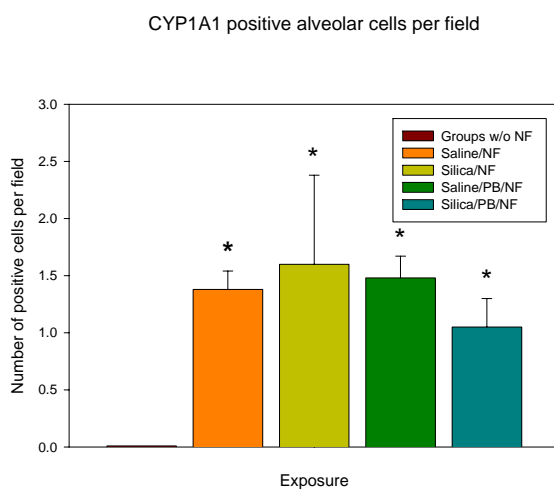


Figure 5. A. Positive non-ciliated airway epithelial cells were only found in groups exposed to  $\beta$ -naphthoflavone. B. Positive plump alveolar epithelial cells (➡) were also found only in groups exposed to  $\beta$ -naphthoflavone. Plump alveolar cells without immunoreactivity (➡) were also found in silica exposed animals. C. CYP1A1 positive cells were present after  $\beta$ -naphthoflavone exposure and the number of cells was not significantly increased by phenobarbital or silica. \*Significantly different from all groups that did not receive NF ( $p < 0.05$ ).

### **3B - The effect of silicosis on induction of CYP-dependent enzymatic activity**

Both ECOD (CYP-dependent) and EROD (CYP1A1-dependent) activities were significantly increased by NF exposure ( $p < 0.0001$ ). The induction of EROD and ECOD activity by BNF was significantly inhibited by prior intratracheal administration of silica (Figure 6).

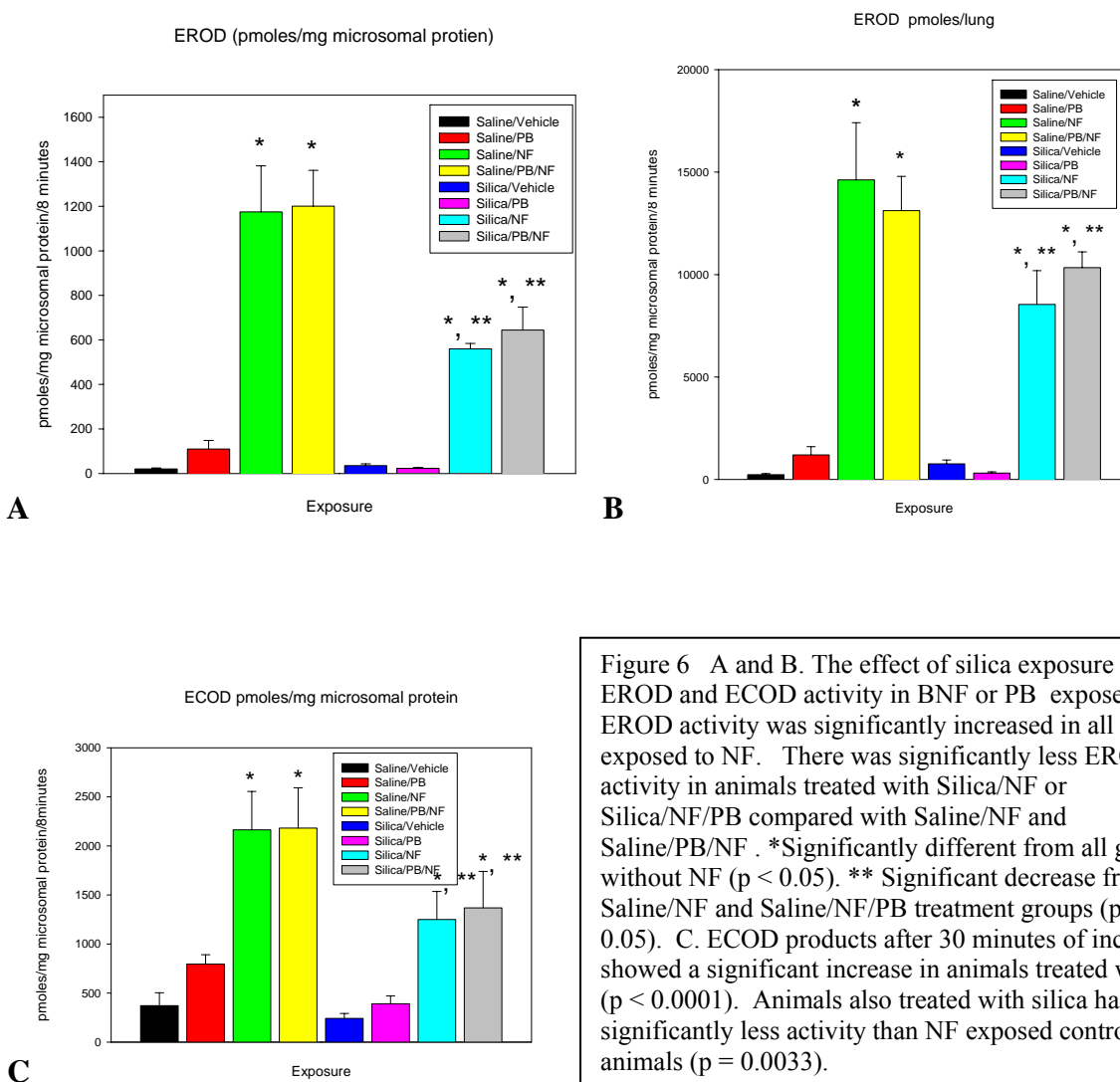


Figure 6 A and B. The effect of silica exposure on EROD and ECOD activity in BNF or PB exposed rats. EROD activity was significantly increased in all animals exposed to NF. There was significantly less EROD activity in animals treated with Silica/NF or Silica/NF/PB compared with Saline/NF and Saline/PB/NF. \*Significantly different from all groups without NF ( $p < 0.05$ ). \*\* Significant decrease from Saline/NF and Saline/NF/PB treatment groups ( $p < 0.05$ ). C. ECOD products after 30 minutes of incubation showed a significant increase in animals treated with NF ( $p < 0.0001$ ). Animals also treated with silica had significantly less activity than NF exposed control animals ( $p = 0.0033$ ).

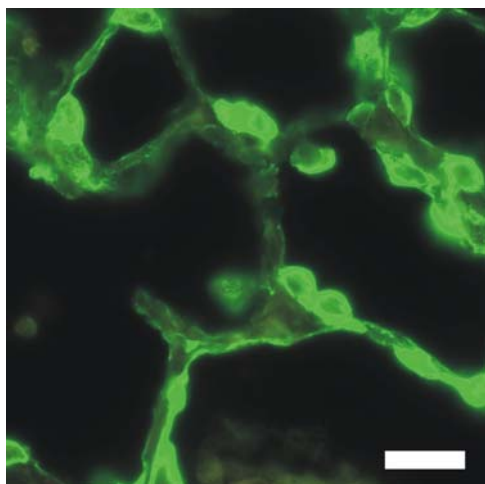


#### **4A - Experiment 2: The effect of silicosis on localized expression of CYP1A1 and Cytokeratin 8 in the pulmonary alveolus of BNF exposed rats**

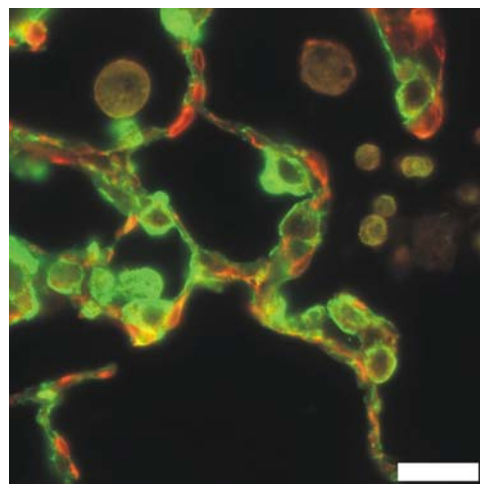
Two distinct areas of the lung were analyzed, the proximal alveolar region (PAR) and the random alveolar region (RAR). The proximal alveolar region is the area immediately adjacent to the terminal bronchi and the random areas are sampled as alveolar regions between visible terminal bronchi. In both the RAR and PAR alveolar regions, CYP1A1 was not observed in the lungs of control rats and intense red fluorescence consistent with induction of CYP1A1 was seen in all rats receiving i.p. NF. Both control and silica-treated rats had plump, cuboidal cells that were morphologically consistent with alveolar type II cells and fluoresced green, indicating cytochrome 8 expression.

##### **The Proximal Alveolar Region:**

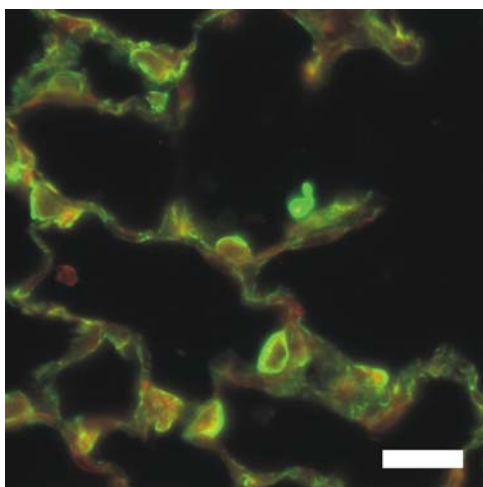
Cytokeratin 8 was expressed in all groups. However, expression was significantly increased in groups exposed to silica. CYP1A1 was only detected in animals exposed to NF (Figures 7 and 8). The amount of CYP1A1 expressed in type II cells was not found to be significantly different between saline and silica groups treated with NF. The proportional expression of CYP1A1 in type II cells (the amount of CYP1A1 expressed in type II cells corrected for increases in type II cell number) was found to be significantly reduced in rats exposed to both NF and silica compared to NF-exposed rats without silicosis (Figures 7 and 8).



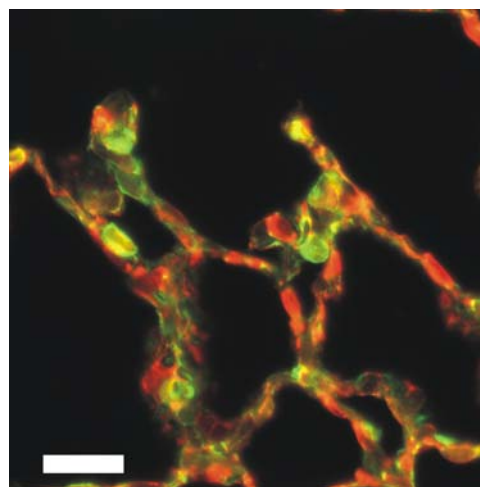
A. Silica/Corn oil



B. Silica/NF



C. Saline/Corn Oil



D. Saline/NF

Figure 7 Immunofluorescent localization of CYP1A1 and Cytokeratin 8 in Proximal Alveolar Regions. Animals that did not receive NF, did not express any detectable CYP1A1. A. In silica/corn oil exposed animal, many plump, cuboidal, cytoke­ratin 8 positive(green), alveolar type II cells are found. B. In silica/NF exposed animals, notice the plump, cuboidal, cytoke­ratin 8 positive cells(green) are abundant, while long, thin, alveolar cells are CYP1A1(red) positive. Yellow staining represents cells expressing both cytoke­ratin 8 and CYP1A1. C. A saline/corn oil exposed animal has far less cytoke­ratin 8 positive (green) cells, and these cells are not as hypertrophied. In addition, CYP1A1 expression is not observed. D. In a saline/NF exposed animal, there are again far fewer and smaller cytoke­ratin 8 positive cells, and CYP1A1 expression is localized in the thin, long alveolar cells. Notice that there are more CYP1A1 positive cells in the septum of saline and NF exposed animal than the silica and NF exposed animal.

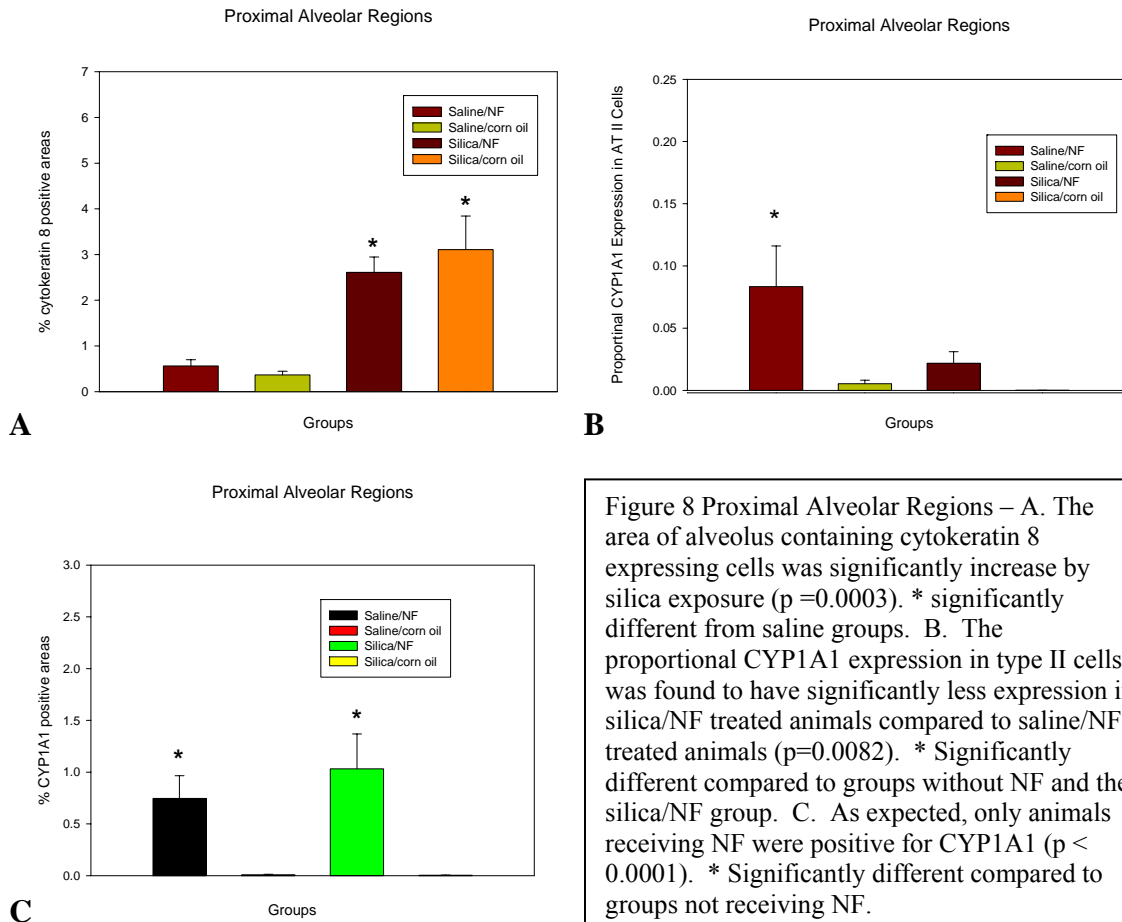
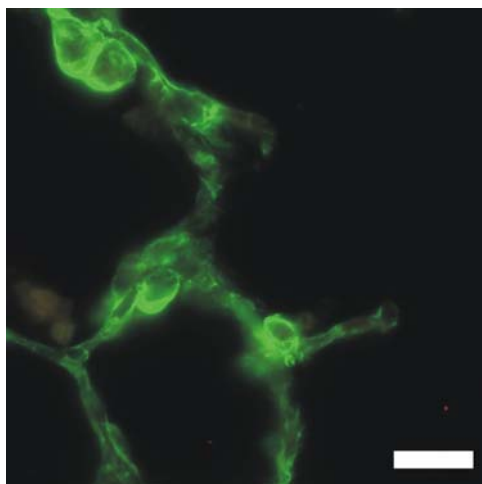


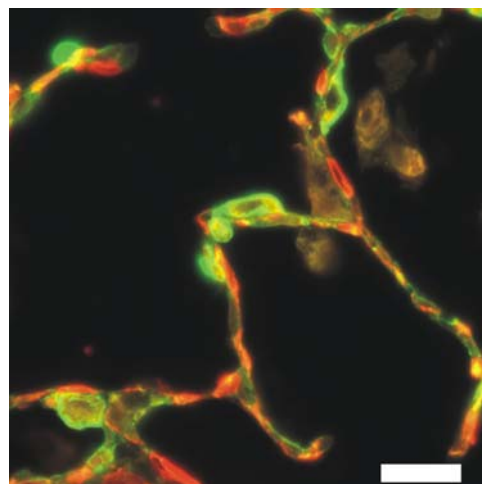
Figure 8 Proximal Alveolar Regions – A. The area of alveolus containing cytokerin 8 expressing cells was significantly increase by silica exposure ( $p=0.0003$ ). \* significantly different from saline groups. B. The proportional CYP1A1 expression in type II cells was found to have significantly less expression in silica/NF treated animals compared to saline/NF treated animals ( $p=0.0082$ ). \* Significantly different compared to groups without NF and the silica/NF group. C. As expected, only animals receiving NF were positive for CYP1A1 ( $p < 0.0001$ ). \* Significantly different compared to groups not receiving NF.

### Random alveolar region:

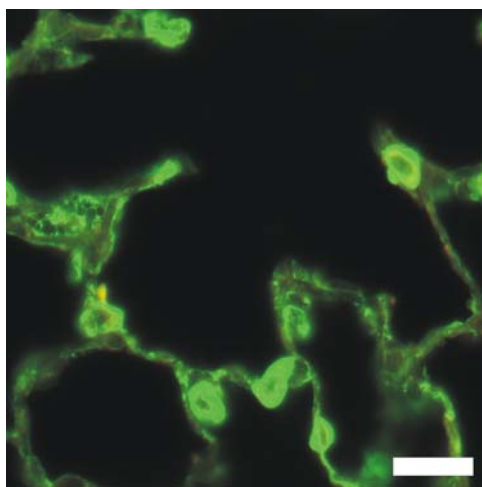
CYP1A1 expression was consistently observed in NF exposed animals. Cytokeratin 8 was expressed in all groups but there was not a significant difference in expression between groups. As in the proximal alveolar region groups, there was not a significant difference in the area of CYP1A1 expressed in either type II cells or non type II cells. The proportional expression of CYP1A1 in type II cells was decreased in silica/bnf treated groups compared with saline/nf groups, but this difference was not statistically significant (Figures 9 and 10).



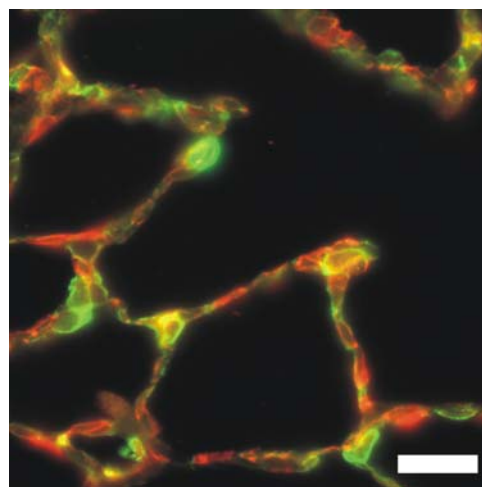
A. Silica/Corn Oil



B. Silica/NF

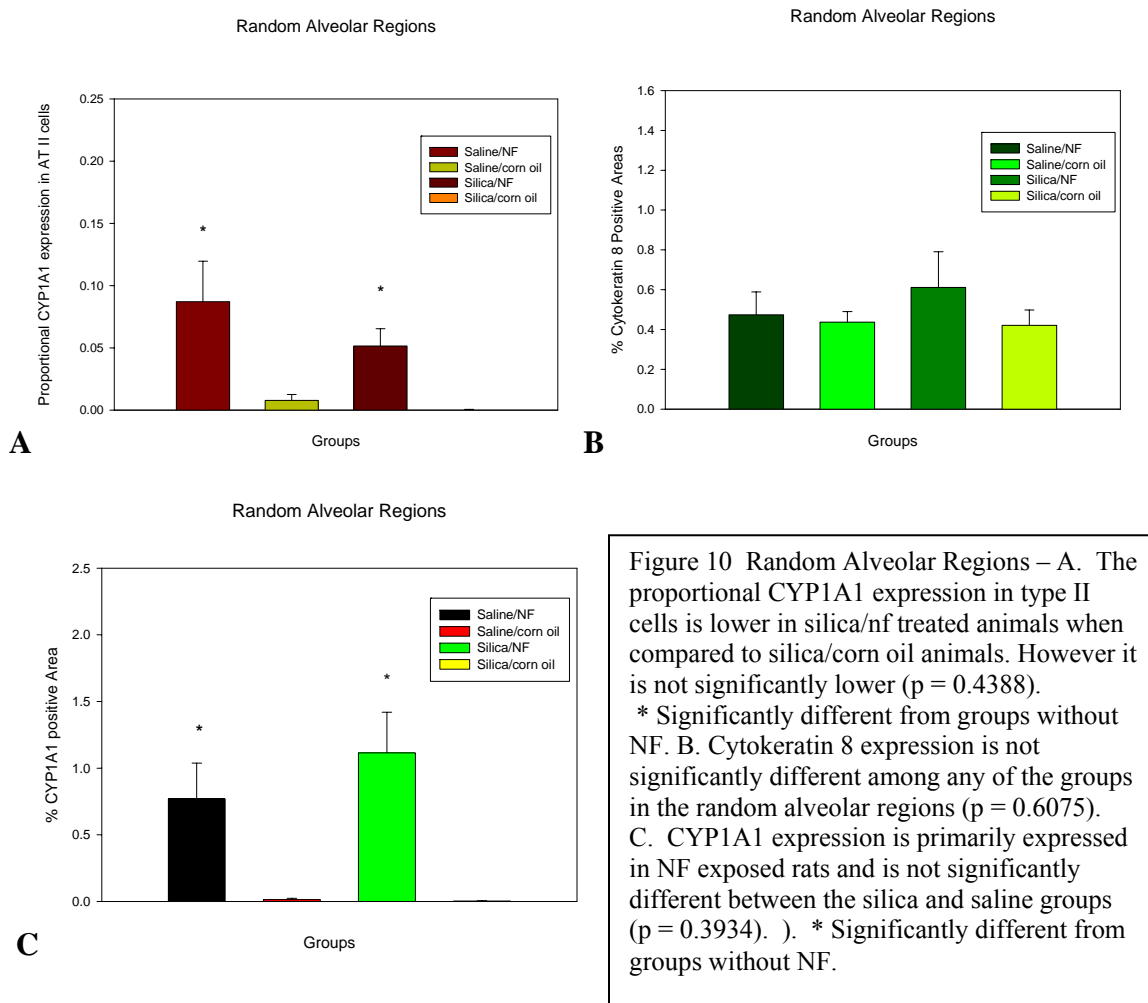


C. Saline/Corn Oil



D. Saline/NF

Figure 9 Immunofluorescent localization of CYP1A1 and cytokeratin 8 in Random Alveolar Regions. Animals exposed only to saline (A) had comparable expression of cytokeratin 8 in the random alveolar regions as compared with silica exposed animals (C). Animals that did not receive NF did not express CYP1A1. Animals exposed to NF and either saline (B) or silica (D) expressed CYP1A1, but expression was not significantly different in the random alveolar regions.



#### **4B - The effect of silicosis on induction of CYP1A1 and CYP2B1-dependent enzymatic activity**

CYP1A1 dependent EROD activity was significantly increased in groups that received NF. There was also a significant decrease in EROD activity of silica/NF groups compared to saline/NF groups. PROD (CYP2B1 dependent) activity was also found to be significantly lower in groups exposed to silica when compare to saline groups. NF treatment did not have a significant effect on PROD activity (Figure 11).

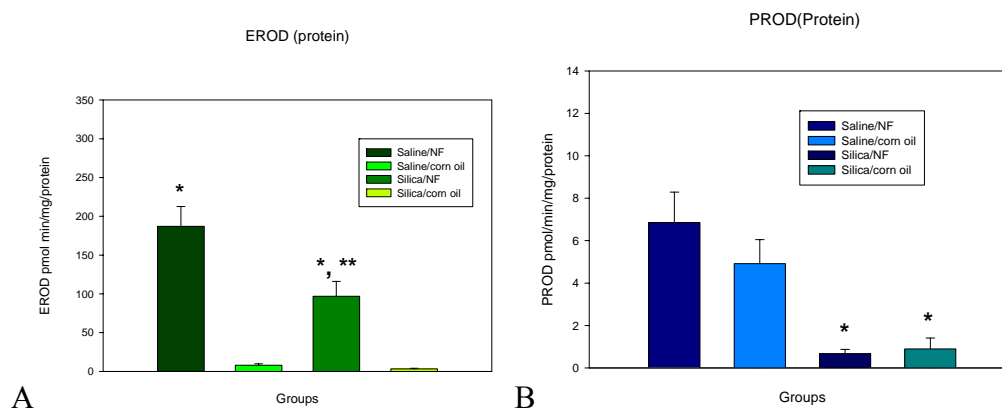


Figure 11 A. EROD (CYP1A1 dependent) activity is significantly increased in groups that received NF(\*) ( $p < 0.0001$ ), and there is a significant decrease in activity in CYP1A1 induction by NF in silicotic rats (\*\*) ( $p = 0.0153$ ) groups compared to saline/nf groups. B. Groups receiving silica had significantly less PROD (CYP2B1) activity than groups than saline groups ( $p < 0.0001$ ). NF did not have a significant effect on PROD activity.

#### **4C - The effect of silicosis on CYP1A1 protein**

Western blot analysis was performed for CYP1A1. As with immunofluorescence, groups that did not receive NF, did not express detectable CYP1A1 protein. For groups receiving NF, the % of positive control was significantly less in silica groups compared to saline groups (Figure 12).

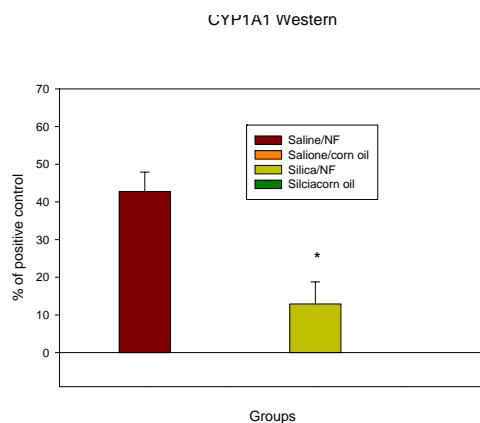


Figure 12 Western Blot analysis of CYP1A1. CYP1A1 was only detected in groups that received BNF. When expressed as percent of positive control, there was significantly less protein expressing in silica/nf groups than saline/nf groups (\*) ( $p = 0.0009$ ).

### **5 - Experiment 3:Silica Dose Response Study with Exposure to Classical Inducer of CYP1A1: The effect of silicosis on induction of CYP1A1 and CYP2B1-dependent enzymatic activity**

BNF induced CYP1A1 dependent activity (EROD) was significantly lower in all silica exposed groups compared to the saline group. There were no significant differences between silica exposed groups. CYP2B1- dependent PROD activity also showed significant decreases in activity of all silica groups compared to saline. There also was not a significant difference between the silica groups (Figure 13).

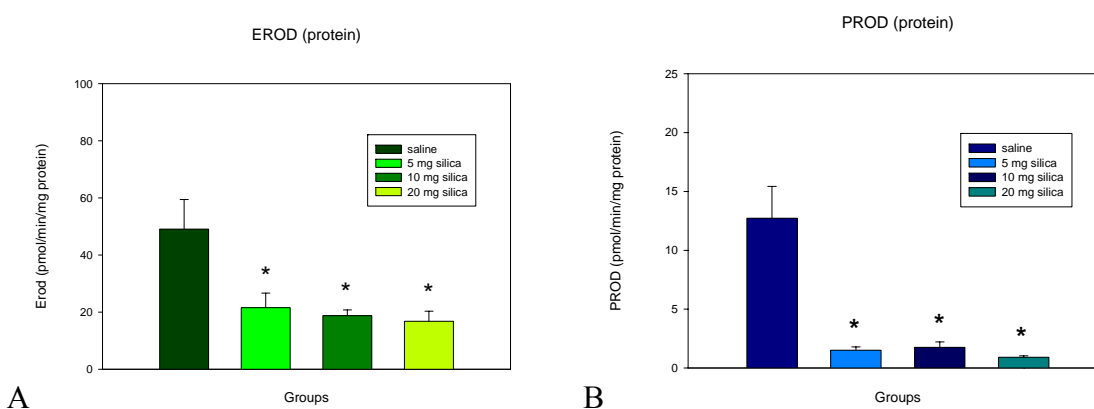


Figure 13 For both PROD (CYP2B1 dependent) and EROD (CYP1A1 dependent) activity was significantly lower in all BNF and silica exposed groups (\*) compared to the BNF and saline exposed group. (A.  $p < 0.0001$ , B.  $p = 0.0035$ ).

### **5A - The effect of silicosis on CYP1A1 and CYP2B1 proteins**

By western blot analysis, silica and BNF exposed groups have significantly less CYP1A1 protein than the BNF and saline exposed group.

Western Blot analysis of CYP2B1 found all BNF and silica groups have significantly less CYP2B1 protein than the BNF and saline exposed group, but there were no significant differences amongst the silica treated groups(Figures 14 and 15).

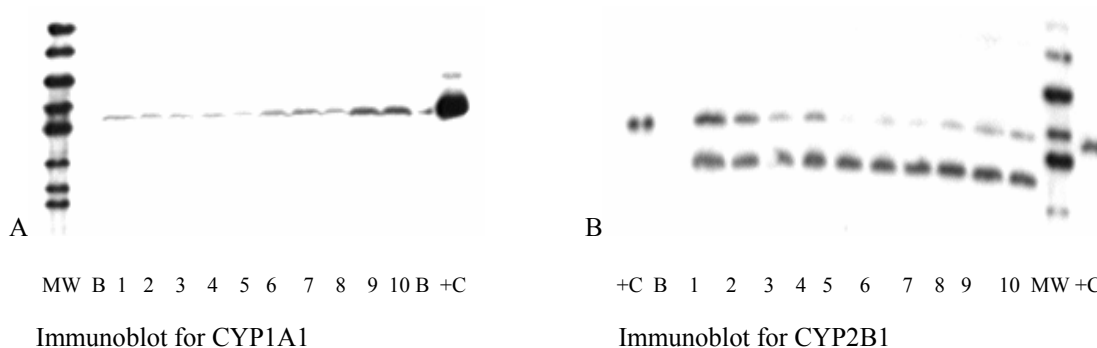


Figure 14 Lanes marked MW stand for Molecular Weight marker, B stands for blank, and +C stands for positive control (BNF treated rat liver microsomes for CYP1A1 and Phenobarbital treated liver microsomes for CYP2B1) For this experiment, all animals received NF. A is an Immunoblot for CYP1A1 – Lanes 1 and 2 were rat lung microsomes treated with 20mg silica, Lanes 3 and 4 received 10 mg silica, lanes 5, 6, and 7 received, 5mg silica, and lanes 8, 9, and 10 received saline. B is an immunoblot for CYP2B1 – Lanes 1, 2, 3 received saline, lanes 4 and 5 received 5 mg silica, lanes 6, 7, and 8 received 10 mg silica, and lanes 9 and 10 received 20 mg silica. In both blots, silica treated animals expressed less protein (CYP1A1 and CYP2B1) than saline treated rats.



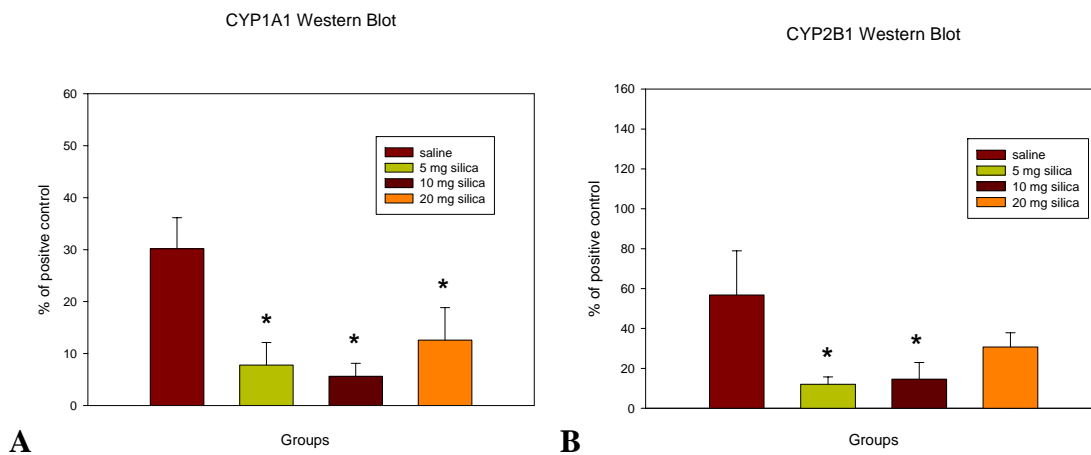


Figure 15 A. Graph quantifying western blots of CYP1A1 protein expression. BNF and silica exposed rats (\*) expressed significantly less CYP1A1 protein than rats exposed to BNF and the IT vehicle (saline) ( $p = 0.0039$ ). B. All BNF and silica exposed rat groups expressed less CYP2B1 protein than rats exposed to BNF and the IT vehicle (saline); however only doses of 5 mg and 10 mg (\*) were significantly different.

## Chapter 5

### Discussion

It is important to note that these experiments were carried out on animals exposed to  $\beta$ -naphthoflavone, a CYP1A1 inducer. EROD and ECOD activity in rats only exposed to saline or silica in the first experiment were found to be comparable with results from Miles et al, 1993 and 1994. EROD activity was increased in animals exposed to silica compared to saline. Levy *et al*, 1997 performed immunohistochemistry for CYP1A1 and CYP2B1 in the lungs of rats that were only exposed to silica or saline. Again, looking at only saline or silica exposed animals from our first experiment, our results were comparable. CYP1A1 was not detected in any animals not receiving NF and although CYP2B1 was detected, there was not a significant difference in the number of cells expressing CYP2B1 between the silica and saline exposed animals. Differences in CYP1A1 activity were only found when animals were exposed to CYP1A1 inducers with and without silica exposure. In NF-exposed rats, silica caused a decrease in CYP1A1 activity, CYP1A1 protein expression, and proportional expression in epithelial type II cells in animals exposed to silica and inducer.

In this study a technique a technique was developed for immunofluorescent labeling and quantifying of alveolar type II cells that was time efficient and allowed for ease of data quantification. Previous research on alveolar type II cell proliferation has been performed using electron microscopy techniques that were time consuming and costly and required special fixation (Miller and Hook, 1990). In 1987, Miller *et al* described a technique using alkaline phosphatase immunohistochemistry that was a vast

improvement over electron microscopy. However, with this technique, fixation and the immunohistochemistry procedure must be carried out at 4 °C to preserve enzyme activity (Miller, *et al* 1987). In the alveolar regions, only epithelial type II cells stained positively for alkaline phosphatase making them easy to recognize under a microscope. However, photomicrographs had to be taken and someone counted the number of positive cells.

Although this was more cost efficient and less time consuming than electron microscopy, our method had advantages which included simplified tissue preservation, technical simplicity and rapid quantitative analysis. Using our method, the lungs could be fixed at room temperature with 10% neutral buffered formalin overnight and embedded in paraffin. They could be stored in paraffin for at least a year prior to analysis.

Immunofluorescent staining was simple relative to alkaline phosphatase immunohistochemistry. Staining for cytokeratin 8 did not prevent subsequent staining with different fluoroprobes which permitted staining of 2 or more different antigens in a single tissue section. Using a fluorescent microscope with an attached digital capture allowed for instantaneous capture of the image. The instantaneous capture prevented the fluorescent fading which has long been a negative consideration with immunofluorescence (Johnson *et al*, 1982). The immunofluorescent images could then be saved onto a disk and the image could be analyzed by a morphometry program.

Morphometry programs quickly quantified the amount of a fluoroprobe present in an image. Numerical area values for area of fluorochrome staining, and percent of the total area staining with a fluorochrome, and colocalization of multiple fluorochromes were easily determined and loaded into analytical programs. Using IF with quantitative morphometry, slides were rapidly analyzed for alveolar type II cell proliferation. In

addition, we localized the expression of CYP1A1 in relation to the sites of cytokeratin 8 expression.

We demonstrated that the proximal alveolar region was the site of specific cell changes. We were able to show proliferation of alveolar type II cells in this region in response to silica exposure. We also were able to show that proportional CYP1A1 expression decreased in animals exposed to both silica and the NF inducer when compared to animals exposed to just NF. This was an important region to analyze as it was the site of particle deposition in intratracheally instilled rats (Brain *et al*, 1976). Inhalation methods of exposure resulted in deeper deposition of particles and may have produced different results. However, we were able to use our technique for detecting alveolar epithelial cell hypertrophy and hyperplasia and localizing CYP1A1 cellular expression and identified the site of particle deposition as the site where expression changed.

Smoking status is a critical factor in lung cancer development and should be a controlled variable in epidemiological studies of lung cancer involving other potential pulmonary carcinogens. However, one epidemiologic study suggested that people exposed to both silica and cigarette smoke had no difference in risk or even a reduced risk for lung cancer compared to people exposed to silica alone (Cocco, *et al* 1991). Many epidemiological studies either did not control, or inadequately controlled for cigarette smoke exposure. Pairon *et al*, 1991, reported that in some studies, smoking was not used as a variable at all. Although IARC added silica to its list of human carcinogens in 1997, some studies have not yet listed silica as a carcinogen (Tsuda *et al*, 2002) and some studies have disputed the carcinogen designation by IARC (Graham *et*

*al*, 2004). Our studies showed that the model, PAH, NF was not a co-variable, but a modifier of lung cancer risk. Thus, it may be appropriate to analyze data from potential lung carcinogens both with control for cigarette smoking and with separate analyses for potential carcinogenesis in smokers and non-smokers.

In vivo and in vitro experiments have shown that inflammation decreases CYP1A1 activity (Tian *et al*, 1999; Ke *et al*, 2001; Paton and Renton, 1998) and inflammation was produced by silica exposure (Porter *et al*, 2001; Driscoll *et al*, 1991; Castranova *et al*, 2002). Several cytokines involved in lung inflammation, such as TNF- $\alpha$  and NF $\kappa$ B interfered with CYP1A1 induction (Tian *et al*, 1999; Ke *et al*, 2001; Paton and Renton, 1998). Experiments by Porter *et al* (2001), showed increases in both TNF $\alpha$  and NF $\kappa$ B in alveolar lavage fluid in rats exposed to silica. NF $\kappa$ B was shown by Tian *et al* (1999) to interact with the Ah receptor. This interaction was a possible reason for the CYP1A1 inhibition by cytokines. The binding to the XRE by the AhR and ARNT produced increased histone acetylation, an important activator of gene transcription (Ke *et al*, 2001). TNF- $\alpha$  treatment inhibited this acetylation and inactivated the CYP1A1 promoter, thereby inhibiting CYP1A1 induction (Ke *et al*, 2001). Cytokines like TNF- $\alpha$  are released in response to inflammatory injuries such as those caused by exposure to silica could possibly explain the decrease in CYP1A1 activity and expression in rats exposed to inducers of CYP1A1 and silica. Our studies showed that down regulation of CYP1A1 expression and activity occurred *in vivo*, in the rat lung after exposure to silica, a known inducer of inflammation. We confirmed inflammation by histopathology in our study

Future studies might include extending the time period after intratracheal exposure. The animals in these studies were sacrificed 14 days after receiving a bolus intratracheal instillation of silica. It would be interesting to see if the significant changes found in the proximal alveolar region would still be present at 4 weeks or more following instillation. Another consideration to explore would be to administer the silica via inhalation exposure. Inhalation is considered a more natural method of exposure but costs and time would increase. Since inflammation was observed in our study and has also been demonstrated following inhalation exposures (Driscoll *et al*, 1991, Castranova *et al*, 2002, Porter *et al*, 2001, Castranova *et al*, 1996 ) the acute effects of silica on down regulation of CYP1A1 expression are likely to be unaffected by the method of exposure.

Exposure to 5 milligrams of silica was sufficient to inhibit CYP1A1 or CYP2B1 expression and activity. Histopathological changes associated with silica exposure (alveolitis, alveolar type II cell hyperplasia and hypertrophy, and lipoproteinosis) were also fully manifested in the lungs of rats at exposures of 5 mg silica. Perhaps exposing the animals to even smaller amounts of silica or allowing for larger increases between doses (i.e., 5, 25, and 50 mg treatments) would show a dose response effect. In our study, histopathologic manifestations of inflammation were also unaffected by the dose at this time point. Thus, doses or time points that changed inflammation would be more revealing in evaluating the relationship between silica-induced inflammation and down-regulation of CYP1A1 activity and expression.

## Conclusions

- Xenobiotic metabolism is altered by silica exposure and is localized to a common site for deposition of silica and other respirable particles, the proximal alveolar duct region.
- These findings suggest that silica exposure modifies carcinogenic effects of PAHs, such as these in cigarette smoke.
- The proximal alveolar region is very important for inhaled carcinogens.
- Silica exposures of 5, 10, or 20 mgs each significantly decreased CYP1A1 and CYP2B1 activity in NF-exposed rats, but a dose response relationship was not detected.

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## VITA

### Personal Information

Name: Lori Ann Battelli  
Address: 643 Tyrone Avery Road  
Morgantown, WV 26508  
Phone: 304-594-2345

### Education

B.S. Pennsylvania State University, Dairy and Animal Sciences 1991  
  
RLAT American Association of Laboratory Animal Science, Technician Certification 1997  
  
M.S. West Virginia University, Genetics and Developmental Biology 2004

### Professional Positions

Research Biologist 1999 - present  
National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Pathology and Physiology Research Branch, Morgantown, WV  
  
Biological Laboratory Technician 1996 - 1999  
National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Pathology and Physiology Research Branch, Morgantown, WV  
  
Necropsy Group Supervisor 1992 - 1996  
MPI Research  
Mattawan, MI 49076

### Professional Societies

Allegheny-Erie Society of Toxicology (AESOT) 1999 – present  
Allegheny-Erie Society of Toxicology(AESOT), Counselor 2004  
American Association for Laboratory Animal Science (AALAS) 1994 - present  
Three Rivers Branch AALAS, Newsletter Editor 1999 – present

### Awards

Charles C. Shepard Science Award Nominee, Outstanding Science Paper 2004  
Alice Hamilton Award, Honorable Mention 2002  
On the Spot Award: March 1997, May 1997, January 1998, April 1999