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Effects of Side-Stream Cigarette Smoke on Murine Cytochrome P450 1A1 Activity

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ABSTRACT

The cytochrome P450 enzyme family is a group of heme-thiolate monooxygenases (E.C. #1.14.4.1) involved in the metabolism of a wide variety of substrates, including xenobiotics, endogenous lipids, and lipid metabolites, to generate potentially cytotoxic compounds. In this study, cytochrome P450 isoenzyme 1A1 (CYP1A1) activity was measured in heart, lung and liver tissues of C57BL/6 female mice exposed to side-stream cigarette smoke from 3 low tar cigarettes per day for up to 6 months. Activity was detected by the cytochrome P450-mediated *O*-dealkylation of ethoxyresorufin to release fluorescent phenoxazone. Cytochrome P450 activity was enriched in the microsomal fractions of liver, lung and heart tissues of control mice. After one month of exposure, the cytochrome P450-mediated *O*-dealkylation of ethoxyresorufin was lower in microsomal fractions from the experimental group relative to microsomal fractions from the control group. The largest differences were observed in lung and heart microsomes which decreased to 73.0% and 65.2% of the corresponding control microsomes, respectively. Lung tissue isolated from mice at two, four and six months of exposure to filtered air showed a 2 to 2.5 fold increase in microsomal-associated cytochrome P450 activity. Mice exposed to side-stream cigarette smoke over the same intervals showed little to no change in cytochrome P450 activity associated with the lung microsomal fraction relative to the activity measured at 1 month exposure. The decrease in lung microsomal-associated cytochrome P450 activity upon exposure to side-stream cigarette smoke is not due to a redistribution of the cytochrome P450 protein among subcellular organelles, but is possibly due to inactivation of the enzyme.

Abbreviations: AHH, aryl hydrocarbon hydroxylase; BSA, bovine serum albumin; CYP1A1, cytochrome P450 isoenzyme 1A1; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; P450, cytochrome P450; SN, supernatant.

INTRODUCTION

We are exposed to a multitude of potentially toxic chemicals on a daily basis. Many of these compounds are chemically inert and only become toxic after they are modified in-

side our cells. One prominent cellular mechanism for the generation of toxic compounds and detoxification of others involves the P450 cytochromes (Porter and Coon, 1991). The P450 cytochromes are ubiquitous proteins found in all organisms, from bacteria to humans, and are present in different amounts in various mammalian tissues (Walker et al., 1995). In cells of higher organisms, P450 proteins are anchored to the microsomal membranes, the site of both lipid metabolism and the detoxification pathways (Black, 1992). In mammals, the highest levels of constitutively-expressed cytochrome P450 are observed in the adrenal tissues and testes (Walker et al., 1995). However, the P450 cytochromes from liver microsomes have been the most extensively studied of the mammalian homologs with regard to catalytic activity and biological function.

Cytochrome P450 is an enzyme that can catalyze either oxidation or reduction of its substrates. Most P450-mediated reactions involve the transfer of electrons from NADPH to molecular oxygen, followed by the insertion of a single oxygen atom into the substrate (Porter and Coon, 1991). The conversion of a hydrocarbon (RH) to the corresponding alcohol (ROH) is one type of reaction catalyzed by P450 to facilitate clearance of the nonpolar xenobiotics. Often free radicals are generated as byproducts of these reactions, and consequently the role of P450 in detoxification is somewhat contradictory (Porter and Coon, 1991). For instance, P450 can convert ethanol, a xenobiotic, to an even more detrimental substance, acetaldehyde.

Cytochrome P450 has been investigated quite thoroughly in the liver, which is attributed to the liver's role in detoxification. However, the lung serves as another organ that is in direct contact with xenobiotics and research indicates that pulmonary tissue may serve as the first site in the biotransformation pathway (Omiecinski et al., 1990). It has been suggested that the regulation of P450 isoenzyme CYP1A1 gene expression in cells, such as pulmonary alveolar macrophages or lung epithelial cells, exposed directly to environmental substances may be a prime determinant of susceptibility to the detrimental effects of chemical exposure (Omiecinski et al., 1990). In this regard, Murray et al. (1997) have shown by immunohistochemical studies that the P450 isoenzyme CYP1B1 localizes to cancerous tissues, including lung and breast. The localization of CYP1B1 to certain tumors may have significant ramifications on cancer treatment.

In this study, CYP1A1 activity was measured in heart, lung and liver tissues of C57BL/6 female mice exposed to side-stream cigarette smoke from 3 low tar cigarettes per day for one month. Activity was detected by the cytochrome P450 mediated *O*-dealkylation of a non-fluorescent phenoxazone ether substrate to release fluorescent phenoxazone. The expression of CYP1A1 activity in lung microsomal fractions was analyzed following daily exposure to side-stream cigarette smoke for up to 6 months.

MATERIALS AND METHODS

Reagents and Animals

Protein binding assay reagents were purchased from Biorad (Hercules, CA). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO). Marlboro Reds™ cigarettes were stored at 4ºC until day of use. Female strain C57BL/6 mice (*Mus musculus)* were purchased from the Trudeau Institute, Saranac Lake, NY. During the course of the experiment, animals were housed, 5 per box, on sterilized wood chips in polypropyl-

ene boxes with wire screen lids. The animals were kept at 23ºC on a twelve hour light-dark cycle and had free access to conventional laboratory rodent chow and water *ad libitum* for the duration of the study.

Exposure of Mice to Cigarette Smoke

In total, twenty 6-8 week old C57BL/6 mice weighing 15 to 20 g were divided at random into two groups. One group of 10 mice was exposed to side-stream cigarette smoke (experimental group) from 3 Marlboro Reds™ cigarettes in one hour (10-11 am) per day for six days a week (2.0 L/min). According to the manufacturer, each cigarette contains15 mg tar and 1.1 mg nicotine. The experimental treatment involved pumping smoke from the lit end of the Marlboro Reds™ cigarette (side-stream or "second-hand" smoke) into an isolated chamber (6.9 dm^3). The control group (10 mice) was treated in the same manner, but fresh air, instead of smoke, was pumped into the chamber at 2.0 L/min for one hour (9-10 am) per day for six days a week. After 1, 2, 4, or 6 months of treatment, two to three mice from each group were sacrificed via carbon dioxide asphyxiation. All animals were sacrificed 48 hours following the final treatment.

Preparation of Samples

Tissue were kept on ice during all stages of sample preparation and maintained between 2 to 6ºC during centrifugation. The hearts, lungs, and livers were harvested from the mice, weighed, and perfused with ice cold 5.0 mM TRIS-HCl buffer(pH7.4) containing 0.25 M sucrose and 10 mM EDTA. Livers, lungs, and hearts from each group were homogenized in 5 volumes of ice cold 5.0 mM TRIS-HCl buffer (pH7.4) containing 0.25 M sucrose, 10 mM EDTA and 10 μM PMSF. The tissue homogenates were subject to differential centrifugation at 4ºC using a Sorvall RC5B centrifuge and a Beckman L8-70M ultracentrifuge. The pellet and a fraction of the supernatant (SN) from each step were collected and analyzed for total protein and cytochrome P450 activity.

Analysis of Samples

Total protein concentrations in tissue homogenates and subcellular fractions were determined using the Bio-Rad Coomassie brilliant blue G-250 protein binding assay based on the method of Bradford (1976). Bovine serum albumin (BSA) served as the protein standard. The activity of CYP1A1 in each sample was measured by the *O*-dealkylation of non-fluorescent phenoxazone ether substrate to release fluorescent phenoxazone (Burke, et al, 1985). Briefly, 100 μg of sample protein in 100 mM phosphate buffer, pH 7.0,was equilibrated at 37ºC for 2 minutes. Reactions, done in duplicate, were started by the addition of a substrate cocktail to give a reaction mixture containing 250 μM NADPH, and 5.0 μM ethoxyresorufin, and were incubated at 37ºC for exactly 10 minutes. Reactions were stopped by the addition of 1.0 mL reagent grade methanol and cooled to room temperature. Fluorescence emission was monitored at 585 nm (sw 10 nm) following excitation of the sample at 530 nm (sw 5nm) using a Perkin Elmer LS50B Luminescence Spectrophotometer. Control reactions, in which protein was replaced with homogenization buffer, contained either substrate cocktail (negative control or blank) or 50 nM resorufin (positive control).

RESULTS

Figure 1 compares the CYP1A1 activity in crude and microsomal fractions following one month of treatment. CYP1A1 activity is presented as a percentage of the activity in the crude liver extract of the control mice group of that study in order to normalize for variations between experiments. The crude tissue extracts show comparable levels of CYP1A1 activity between the experimental and control groups (Figure 1A). Liver exhibited the highest activity in the crude tissue extract (65.5 pmol/min/mg) followed by lung (78% control crude liver CYP1A1 activity) and heart (52-60% control crude liver CYP1A1 activity). The CYP1A1 activity was slightly enriched in the microsomal fractions for all tissues studied due to the removal of non-P450 protein and, perhaps, endogenous CYP1A1 inhibitors by differential centrifugation (Figure 1B). For all tissues, the CYP1A1 activity in the microsomal fractions was lower for the experimental group than the control group. The largest differences were observed in heart microsomes, which decreased to 65.2% of the activity measured in heart microsomes from the control group, and lung microsomes, which decreased to 73.0% of the activity detected in lung microsomes from the control group.

Figure 2 demonstrates the changes in lung microsomal CYP1A1 activity over the six month study. Interestingly, CYP1A1 activity in the lung microsomal fraction of control mice increased dramatically from 161% to 405% control crude liver CYP1A1 activity between one and two months of treatment. Four and six months into the study, lung microsomes of the control group exhibited 306 to 320% of the control crude liver CYP1A1 activity. This increase in CYP1A1 activity in lung microsomal fractions of the control group may be due to development-associated changes in the expression of lung cytochrome P450 enzymes. Alternatively, this discrepancy may be due to insufficient cell membrane disruption during the homogenization process of the samples treated for one month. CYP1A1 activity associated with lung microsomes from the experimental group remained relatively unchanged throughout the study, ranging from 110 to 142% of the control crude liver CYP1A1 activity. At all time points, the CYP1A1 activity associated with the lung microsomal fraction of the experimental group was significantly lower than the CYP1A1 activity associated with the lung microsomal fraction of the control group. The largest differences were observed following two and four months of treatment, each exhibiting a 2.9 fold decrease in CYP1A1 activity between control and experimental groups.

It has been previously demonstrated that exposure of rats and mice to 3-methylcholanthrene, a potent chemical inducer of CYP1A1, resulted in the induction of CYP1A1 in lung tissue as detected immunocytochemically (Lee and Dinsdale, 1995). Furthermore, immunostaining localized the CYP1A1 protein to the cytoplasm of the rodent lung endothelial cells. To examine the possibility that the lung CYP1A1 activity in the mice exposed to side-stream cigarette smoke was mislocalized and not enriched in the microsomal fraction, we analyzed the CYP1A1 activity in all subcellular fractions collected for the 2, 4, and 6 month time points. Figure 3 shows the subcellular distribution of CYP1A1 activity in lung tissue of control and experimental groups following two months of treatment. Subcellular distribution profiles of CYP1A1 activity in lung tissue after 4 and 6 months of treatment are virtually identical (data not shown). While lung CYP1A1 activity is detected in all of the subcellular fractions, the majority of the activity in both the con-

trol and experimental groups sediments predominantly with the microsomal fraction as expected. Enrichment of CYP1A1 activity in the cytosolic fraction (SN 100,000 x g) was not observed.

DISCUSSION

In this study, exposure of mice to side-stream cigarette smoke decreased the activity of CYP1A1 in the lung microsomal fraction. It has been reported, in an epidemiological study, that CYP1A1 expression correlates positively with exposure to environmental cigarette smoke (McLemore et al., 1990). Similarly, Murray et al. (1997) found that the CYP1B1 expression is significantly greater in cancerous tissues than in normal tissues. Clearly, these previous reports contradict the results of this particular study. One possible explanation for this discrepancy centers on the difference in subjects tested. While C57BL/6 mice were subjected to controlled amounts of side-stream cigarette smoke in this experiment, the other projects used humans that possessed different histories of exposure to cigarette smoke. Consequently, these previous reports do not lie within the boundaries of a controlled study as this one does.

Kawamoto et al. (1993) demonstrated that exposure of rats to side-stream smoke from a Japanese brand of cigarettes induced the expression of several P450 isoenzymes in the liver. The increase of isoenzymes CYP1A1, CYP 1A2, and CYP2B1 was detected immunochemically after the five days of exposure to side-stream smoke. Although the levels of the different P450 proteins increased following exposure to side-stream smoke, a significant decrease in aryl hydrocarbon hydroxylase (AHH) activity following exposure to high concentrations of cigarette smoke was observed. Exposure to five Mild Seven cigarettes per hour for five days resulted in a 34.2% decrease in liver AHH activity relative to control. AHH activity is one of the activities associated with the CYP1A1 isoenzyme (Raunio et al., 1983) and was the only P450 activity examined by Kawamoto et al. (1993). Aryl hydrocarbons are known to induce CYP1A1 and CYP1A2 (Bilimoria and Ecobichon, 1980), and cigarette smoke contains many aryl hydrocarbons (Kawamoto et al., 1993), so the immunochemical results were not surprising. However, the effect of cigarette smoke on AHH activity in liver microsomes has been disputed. Some studies have shown an increase in activity upon exposure to cigarette smoke (Raunio et al., 1983), while others have shown no induction (Bilimoria and Ecobichon, 1980). In agreement with our observations in mouse lung, Kawamoto et al. (1993) actually show a decrease in CYP1A1 activity following exposure to high levels of side-stream cigarette smoke. It has been suggested that the activity of the P450 protein was compromised by the polycyclic aromatic hydrocarbons in cigarette smoke without affecting the antigenicity (Kawamoto et al., 1993). In future experiments, we will examine the expression levels of CYP1A1 in murine heart and lung tissues exposed to side-stream cigarette smoke for the experimental time points by both quantitative PCR and immunochemical analysis.

In this regard, induction of CYP1A1 expression has already been demonstrated immunocytochemically in lung capillary endothelial cells from male strain A/J mice exposed to chronic levels of side-stream cigarette smoke for six months (Pinkerton et al., 1996). However, relatedness of these results to our study is uncertain as gender differences have

been observed in the induction of different P450 isoforms in response to *p*-chlorobenzotrifluoride treatment of Sprague-Dawley rats (Pelosi et al., 1998)

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Figure 1. CYP1A1 activity in liver, heart, and lung tissues from mice exposed daily for 1 month to filtered air (light bars) or side-stream cigarette smoke (dark bars). Activity was measured as described in the methods and is expressed as a percentage of the activity detected in crude liver extract of the control group (65.5 pmol/min/mg). Each data point represents the mean ± S.D. (n =2). Panel A compares activity in the crude tissue extracts. Panel B shows the activity detected in the microsomal fraction.

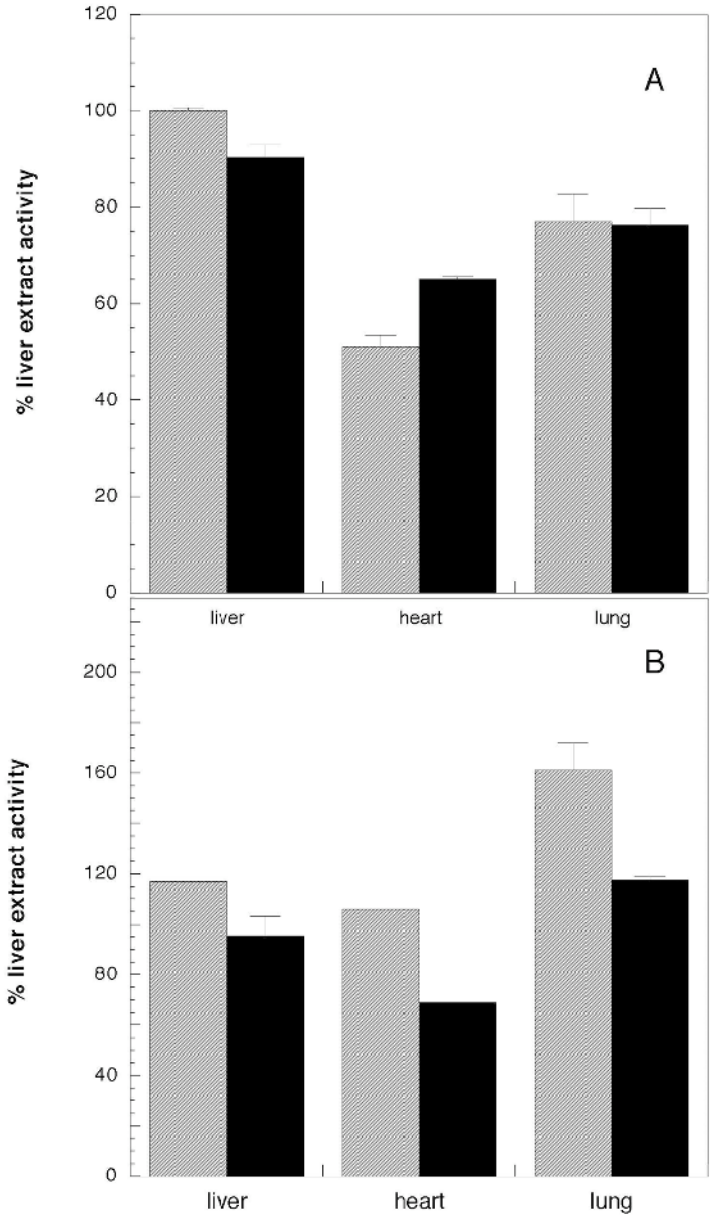

Figure 2. CYP1A1 activity in lung microsomes from mice exposed daily to filtered air (light bars) or side-stream cigarette smoke (dark bars) as a function of duration of exposure. Activity was measured as described in the methods and is expressed as a percentage of the activity detected in crude liver extract of the corresponding control group (65.5 pmol/min/mg-72.1 pmol/min/mg). Each data point represents the mean ± S.D. (n = 2 for 1 and 4 month exposure, n =3 for 2 and 6 month exposure).

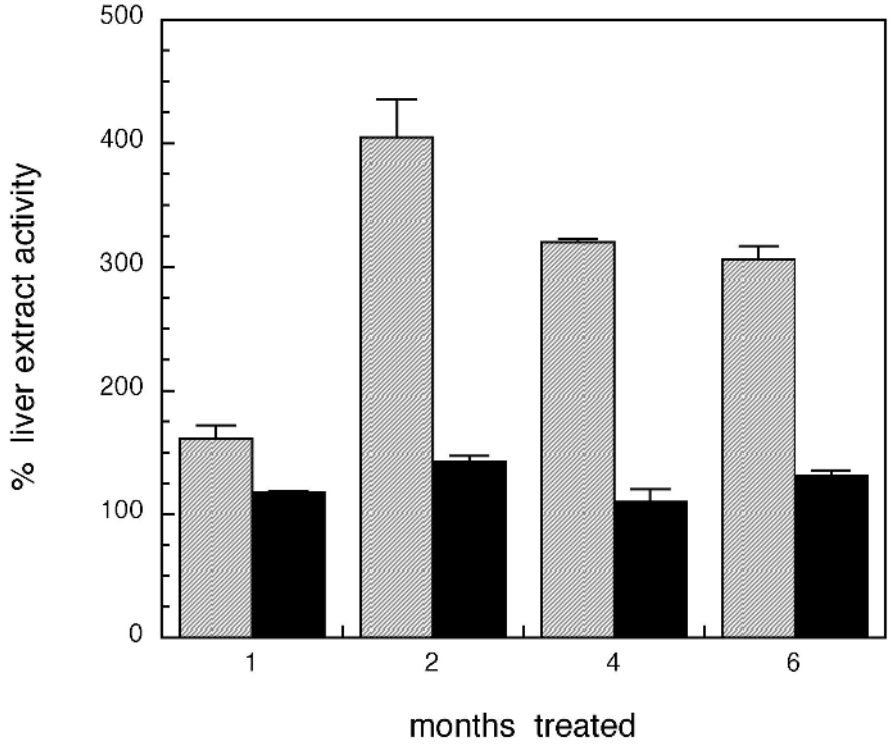

Figure 3. CYP1A1 activity in subcellular fractions of lung tissue from mice exposed daily to filtered air (light bars) or side-stream cigarette smoke (dark bars) for 2 months. Activity was measured as described in the methods and is expressed as a percentage of the activity detected in crude liver extract of the control group (72.1 pmol/min/mg). Each data point represents the mean ± S.D. (n= 3)