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METABOLIC ACTIVATION OF THE PNEUMOTOXIN, 3-METHYLINDOLE, BY VACCINIA-EXPRESSED CYTOCHROME P450s

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SUMMARY: Twelve human cytochrome P450s and one mouse P450 were produced in HepG2 cells using vaccinia virus cDNA expression and analyzed for their ability to bioactivate the pneumotoxin, 3-methylindole (3MI), to an electrophilic metabolite(s) which alkylated cellular macromolecules. Cell lysates containing CYP2C8, CYP3A4, CYP2A6 and CYP2F1 metabolized 3MI to an intermediate(s) that became covalently bound to lysate material. A control lysate produced from cells which had been infected with a wild-type vaccinia virus was not able to bioactivate 3MI. The mouse 1A2 enzyme metabolized 3MI at a rate of 75.4 pmol/mg protein/minute, while the rate of metabolism in the lysate containing the human 1A2 P450 enzyme was not different from that in the control lysate. Therefore, the catalytic capabilities of orthologous P450 enzymes to activate 3MI cannot be extrapolated among different species. These results indicate that human P450s are capable of bioactivating 3MI to a metabolite which binds to cellular macromolecules suggesting that this compound may be toxic to humans.

The discovery over the past few years that certain compounds can selectively or specifically cause lung damage after systemic circulation has spurred interest in the metabolic capacity of this tissue. Studies with 3-methylindole (3MI), a fermentation product of tryptophan produced by anaerobic bacteria in the rumen and large intestine of mammals, indicate that the toxicity of this compound is dependent on bioactivation by cytochrome P450s present in pulmonary tissue (1, 2, 3). In cattle, 3MI causes a disease known as acute bovine pulmonary edema and interstitial emphysema (4). This occurs when cattle are moved from poor quality grazing conditions to lush, green pastures. Stimulated growth of anaerobic bacteria in the rumen results from a disruption in the equilibrium of ruminal microflora. Pneumotoxicity results when ingested tryptophan is

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Abbreviations: 3MI, 3-methylindole; ABT, 1-aminobenzotriazole; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); EDTA, ethylenediaminetetraacetic acid.

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metabolized by ruminant bacteria to indole-3-acetic acid which is subsequently metabolized by anaerobic bacteria, specifically *Lactobacillus* sp. strain 11201, to 3MI (reviewed in 3). 3MI is absorbed through the rumen and circulated systemically and, although the compound must first pass through the liver prior to lung exposure, toxicity is seen only in pulmonary tissues (5). Several studies have suggested that the toxicity associated with 3MI is due to a pulmonary cytochrome P450-generated metabolite (reviewed in 3). Lung slices from goats are capable of metabolizing 3MI to an intermediate that covalently binds to tissue and this metabolism is inhibited by the cytochrome P450 inhibitor, SKF 525A (6). The suicide inhibitor of cytochrome P450, 1-aminobenzotriazole (ABT), has also been shown to inhibit covalent binding of 3MI to proteins in goat lung microsomal incubations (2). Furthermore, ABT inhibits 3MI turnover and covalent binding in human lung microsomes (7). Further evidence for the involvement of P450 is that nonciliated bronchiolar epithelial cells (Clara) and alveolar type II cells, which contain the highest concentrations of P450 enzymes (8), are the most susceptible to 3MI toxicity (9). In addition, ABT prevents the cytotoxicity of 3MI in these isolated cells (9).

Although human pulmonary tissues have been shown to contain low cytochrome P450 content (10), specific P450 forms have not been as well characterized as those in liver (11). However, the prevalence of lung cancer and its association with exposure to polycyclic aromatic hydrocarbon procarcinogens such as those found in cigarette smoke would suggest that the bioactivation processes of the lung may play an important role in pulmonary toxicology. A recent study using a cDNA probe representative of CYP1A1[1] demonstrated a positive association between cigarette smoking and expression of CYP1A1 mRNA in human pulmonary tissue (12). Induction of other P450s has not been shown in human pulmonary tissue.

The research presented here was done to determine which human P450 enzymes might be capable of bioactivating 3MI to an intermediate which covalently binds to cellular macromolecules. Cytochrome P450 enzyme selectivity for 3MI was previously examined in goat lung microsomes using the selective inhibitor, alpha-methylbenzylaminobenzotriazole (2). 3MI was bioactivated to an intermediate that became covalently bound to proteins in this species by the "phenobarbital-inducible" enzymes that are predominantly responsible for benzphetamine oxidation, presumably members of the 2B subfamily. Several recent studies indicate that substrate specificity of orthologous P450s cannot be extrapolated among species (13, 14, 15). Therefore, while the goat offers an excellent model to study the mechanism of pneumotoxicity of 3MI, data from this species cannot be easily extrapolated to humans. In order to better understand human susceptibility to 3MI toxicity, vaccinia-expressed human P450s were used to examine P450 form-specific metabolism of 3MI to a reactive intermediate capable of binding to cellular macromolecules. This approach may provide valuable information about organ-selective toxicity since certain P450s are known to exist predominantly in specific organ systems. Because of the marked organ selectivity of 3MI, particular attention was paid to examining the 3MI bioactivating ability of those P450 enzymes which have been previously reported to be present in pulmonary tissues of various species.

MATERIALS AND METHODS

Chemicals. 3-Methylindole and [^{14}C]3-methylindole (specific activity=12.2 mCi/mmol) were obtained from Sigma Chemical Company (St. Louis, MO). NADPH was obtained from

[1]The cytochrome P450 nomenclature used is based on the recommendations of Nebert et al. (reference #28).

Calbiochem (La Jolla, CA). Protosol was purchased from Dupont (Boston, MA) and Optifluor from Packard (Downer's Grove, IL).

Expression of cDNAs using vaccinia virus. The construction of recombinant vaccinia viruses containing cDNAs representative of human cytochrome P450 enzymes CYP1A2 (16), CYP2A3 (now called CYP2A6) (17),CYP2B7 (now called CYP2B6) (18), CYP2C8 (19), CYP2C9 (19), CYP2F1 (20), CYP3A4 (21), CYP3A5 (21), CYP4B1 (22) and mouse 1A2 (13) have been previously described. Recombinant vaccinia viruses containing *CYP2D6* (23), *CYP2E1* (24) and *CYP3A3* (25) were constructed according to previously published methods (21). The recombinant viruses were used to infect the human hepatoma cell line, HepG2 (ATCC HB 8065). The cells were harvested twenty-four hours after infection, washed with phosphate-buffered saline and frozen at -70 °C.

Determination of P450s involved in activating 3MI to an intermediate that becomes covalently bound to cellular macromolecules. The frozen cells were thawed and sonicated and then subjected to protein determinations using the method of Smith et al. (26). The quantification of covalently bound 3MI metabolite was performed as previously described (2). Reaction mixtures composed of 1.0 mg lysate protein, 200 μM [^{14}C]3MI (specific activity adjusted to 0.5 mCi with unlabeled 3MI) and 1.0 mM NADPH in 0.5 ml sodium phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM EDTA were incubated for 30 minutes at 37°C. The reaction was stopped with 1 ml methanol and the mixture centrifuged at 1300 x g for 15 minutes. The supernatant was removed and the protein was washed repeatedly with methanol and acetone until the radioactivity of the wash solvents reached background levels. The protein was solubilized overnight with Protosol tissue solubilizer, bleached with 150 μl hydrogen peroxide, incubated for 12 hours in the dark and counted using a liquid scintillation counter. To determine the amount of bound [^{14}C]3MI that was not produced from P450 catalysis, concurrent incubations containing all components except NADPH were carried out simultaneously for each lysate. The data are expressed as rates/mg protein. Previous studies (27) have indicated that the cytochrome P450 levels do not differ significantly among the lysates which express the various P450s. The levels of P450 are consistently 13-18 pmol/mg lysate protein, regardless of the particular P450 cDNA used in the construction of the recombinant virus .

RESULTS AND DISCUSSION

Lysates from HepG2 cells which were infected individually with recombinant vaccinia viruses containing human P450 cDNAs were used to examine P450-mediated bioactivation of 3MI to an intermediate capable of covalently binding to the lysate material. A control lysate containing a vaccinia virus without a P450 cDNA insert was also examined for its ability to bioactivate 3MI. Figure 1A illustrates data from experiments using lysates with expressed human P450s whose orthologues have been detected in pulmonary tissues of various species. CYP2F1 and CYP2A6 metabolized 3MI to a covalent-binding intermediate at a rate of 15.3 and 3.9 pmol/mg protein/minute, respectively. 3MI was also bioactivated by CYP4B1, but binding in this lysate was not significantly different from that of the control. CYP2B6 and CYP2E1 were not capable of bioactivating 3MI. Figure 1B illustrates data from lysates containing P450s that have not been reported to be present in pulmonary tissues. Among the extrapulmonary P450 enzymes analyzed, only CYP2C8 and CYP3A4 were capable of bioactivating 3MI. However, the rates for these enzymes were much lower than that seen for CYP2F1 bioactivation of 3MI. 2F1 was capable of metabolizing 3MI at a rate approximately 9 times greater than CYP2C8 and approximately 4 times greater than CYP3A4. In the lysates made from cells expressing CYP2D6, CYP3A3 and CYP3A5, no covalently bound 3MI metabolite was detected. In each experiment measuring P450-mediated bioactivation of 3MI, an incubation containing all components except NADPH was run concurrently. The covalently bound radioactivity observed in these lysates was approximately the same as that generated in the control lysate with or without the addition of NADPH.

Figure 2 compares the ability of mouse and human CYP1A2 to bioactivate 3MI. As shown in Figure 1B, vaccinia-expressed human 1A2 was unable to bioactivate 3MI to an intermediate that

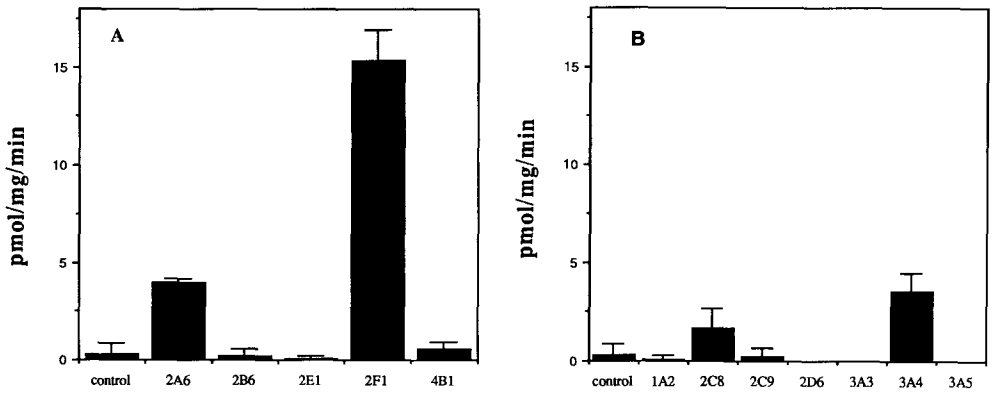

Figure 1. Metabolism of 3MI by (A) vaccinia-expressed pulmonary cytochrome P450s and (B) vaccinia-expressed extrapulmonary P450s to an intermediate that covalently binds to cellular macromolecules. [^{14}C]3MI was incubated with lysates from HepG2 cells which had been infected with recombinant viral vectors containing human P450 cDNAs. The control HepG2 cells were infected with wild-type virus. The forms included in graph A or their orthologues have been identified in the pulmonary tissues of at least one mammalian species. Data represent the mean of at least 3 separate determinations ± standard deviation.

became covalently bound. Conversely, the mouse 1A2 enzyme produced a 3MI reactive intermediate that became covalently bound at a rate of 75.4 pmol/mg protein/min. This is approximately 5 times greater than the rate seen in lysates expressing human 2F1, the human enzyme having the greatest ability to bioactivate 3MI (Figure 1A).

Covalent binding to proteins is believed to be the event initiating the toxic response to 3MI. In studies comparing the covalent binding of 3MI to microsomal proteins from pulmonary and hepatic tissues of goats and cows to that of mice, rats and other non-ruminants, the highest amount of

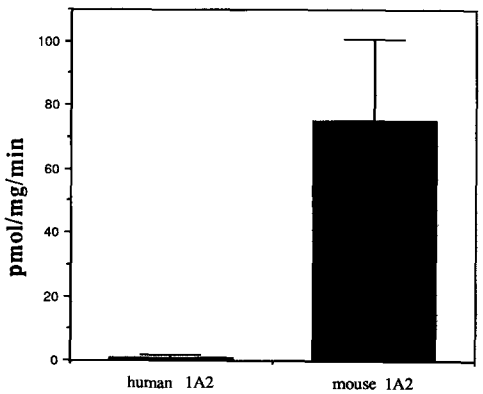

Figure 2. Differences in the bioactivation of 3MI by human and mouse CYP1A2 enzymes.These experiments were performed as described in Fig. 1.

covalent binding was shown to occur in microsomes from the lungs of goats and cows (reviewed in 3). Thus, the covalent binding data was correlated to the species and organ selectivity of 3MI toxicity, since ruminants have been shown to be most susceptible to this compound (3).

The importance of the cytochrome P450 enzyme superfamily in the biotransformation of endogenous, as well as exogenous compounds has been appreciated for a number of years. Recently, more attention has been paid to P450s in extrahepatic tissues. The rabbit pulmonary monooxygenase system has been well studied by Philpot and co-workers. Three P450 forms designated 2, 5 and 6 (by recently proposed nomenclature, CYP2B4, CYP4B1 and CYP1A1(28)) have been isolated (29). CYP4B1 has also been detected in the pulmonary tissues of mice, rats, pigs and monkeys, but not in hepatic tissues of these species (30). CYP4B1 was also isolated from a cDNA library constructed from human lung mRNA.

The P450 enzymes of rat lungs (P450 content about 50 pmol/mg protein) are less thoroughly studied. Ethanol has been shown to increase the metabolic activation of N-nitrosodimethylamine in rat pulmonary microsomes (31) and this is believed to be due to increases in CYP2E1 induced by ethanol. de Waziers and co-workers (32) have also detected this form in rat lung tissue by immunochemical methods. Immunochemical studies have also indicated the presence of CYP1A1, CYP2B1 and CYP3A2 (33). In addition to 2B1, Imaoka and Funae (34) demonstrated the presence of a P450 L-2, which appears to be a member of the 4B subfamily. In rats, CYP2A3 is expressed in pulmonary but not hepatic tissue and is induced by 3-methylcholanthrene (35). A related form (CYP2A6) was isolated from a human hepatic cDNA library and is responsible for coumarin 7-hydroxylase activity in human liver (17). CYP2A6 has been shown to be capable of bioactivating aflatoxin B1 to a mutagenic metabolite (28, 36).

The P450 content in human lung microsomes is approximately 1.2 pmol/mg protein as compared to 225 pmol P450/mg protein in rabbit lung microsomes (10). Cytochrome P450s expressed in human lung tissue are less well-characterized. A cDNA encoding a new P450, designated CYP2F1, was recently isolated from a human lung lambda-gt11 library (20). The mRNA representative of this form was found in low abundance in several human lung samples, but was found in detectable concentrations in only one human liver sample. When the CYP2F1 cDNA was expressed in HepG2 cells using a vaccinia expression vector, the enzyme was able to metabolize several common P450 substrates, but had the highest activity towards ethoxycoumarin (20). However, no correlation was found between this activity and 2F1 mRNA levels in human lung tissue, probably because 2F1 may not be the only form of P450 which metabolizes this substrate. An mRNA transcript hybridizing with CYP2F1 was also detected in rat lung. A P450 responsible for bioactivating naphthalene was purified from mouse lung tissue and amino acid sequence data indicate that this form is the mouse orthologue of human 2F1[2]. The presence of CYP2F1 in human pulmonary tissues indicates that 3MI may be bioactivated to a reactive metabolite in human lung tissue. Humans are subjected to substantial exposure to 3MI, the most predominant route being the metabolism of tryptophan by intestinal microflora. However, 3MI is present in cigarette smoke at concentrations that exceed that of benzo(a)pyrene by a factor of 500 (37). Additionally, 3MI is a component of coffee (38), certain cheeses (39) and seafood (40).

Three human hepatic P450s were also capable of metabolizing 3MI to an intermediate with cellular macromolecule-binding properties. Cytochrome P450 2A6 is present in human hepatic tissue and its expression exhibits a large degree of interindividual variation, with levels varying up

[2]A. Buckpitt, personal communication.

to 40-fold among human microsomal samples (17). While it is not known if this difference is due to a genetic polymorphism, this could be significant with regard to individual susceptibility to toxicity of ingested 3MI. CYP2C8 and CYP3A4 also produced intermediates capable of covalently binding to lysate protein. These data suggest that in humans, hepatic tissue could be a target of 3MI toxicity. Hepatic toxicity is consistent with studies comparing the metabolism and covalent binding of 3MI in human tissues. In microsomes from 8 human lung samples and one liver sample, a 3MI metabolite was covalently bound to protein in a cytochrome P450-dependent fashion in both tissues (7). However, the extent of covalent-binding was 20-fold greater in human liver microsomes than in pulmonary microsomes.

The highest activation of 3MI was produced by the cDNA-expressed mouse P450 1A2 (Fig.2). CYP1A2 is of interest because of its ability to catalyze the N-hydroxylation of arylamines (41). Differences in substrate specificity for human 1A2 and the mouse orthologue have previously been demonstrated (23). Mouse 1A2 catalyzed ethoxyresorufin O-deethylation at a 7-fold higher rate than cDNA-expressed human IA2. Also, mouse 1A2 was 5 to 7-fold more active than the human 1A2 orthologue for activation of 2-acetylaminoflourene and benzo[a]pyrene-7,8-dihydrodiol. However, activation of the food mutagen, dimethyl IQx, was 40% greater for human 1A2 as compared to the mouse orthologue. 3MI is only moderately toxic to mice, although it does display the same characteristic organ-selective toxicity in the mouse as seen in ruminants (42). Interestingly, [^{14}C]3MI administered by intraperitoneal injection to mice produced about 4-fold higher amounts of covalently bound intermediates to liver than lung proteins in spite of the fact that 3MI is highly selective for lung damage in this species (43). This covalent binding to hepatic tissues may be due largely to the substantial activity of the 1A2 isozyme for 3MI metabolism. Since 1A2 has not been reported to be present in mouse extrahepatic tissues, the metabolite formed by this P450 is most likely not responsible for the pneumotoxicity elicited by this compound. Explanations for the pneumotoxic response rather than a hepatotoxic response in mice have not been determined. It may be that the metabolite(s) produced in the pulmonary tissue is more toxic than the one produced in hepatic tissue or that detoxification or conjugation enzymes of the liver, capable of ameliorating any toxicity in this organ, are not present or are not as active in pulmonary tissue as in hepatic tissue. Another explanation may be that certain cell types of the lung may be more susceptible to damage by 3MI metabolites than hepatocytes.

This study indicates that 3MI is bioactivated by P450 enzymes which are present in human tissues to a metabolite capable of covalently binding macromolecules. 3MI metabolites have been identified in human urine and have been associated with a malabsorption syndrome, anemia and Heinz body formation (44). The results of the present study do not permit association of 3MI toxicity with a specific pathological condition in humans, but do suggest that this chemical may be a potential human toxin. However, other factors involved in the manifestation or amelioration of a toxic response, such as detoxification processes and pharmacokinetic factors, must be further investigated in order to fully understand the effects of 3MI on humans.

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