

Metabolic Activation of 4-Ipomeanol by Complementary DNA-expressed Human Cytochromes P-450: Evidence for Species-specific Metabolism

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ABSTRACT

4-Ipomeanol is a pulmonary toxin in cattle and rodents that is metabolically activated by cytochromes P-450 (P-450s). P-450-mediated activation of 4-ipomeanol to DNA binding metabolites was evaluated using a vaccinia virus complementary DNA expression system and an *in situ* DNA-binding assay. Twelve human P-450s and two rodent P-450s were expressed in human hepatoma Hep G2 cells and examined for their abilities to metabolically activate this toxin. Three forms, designated CYP1A2, CYP3A3, and CYP3A4, were able to catalyze significant production of DNA-bound metabolites of 20-, 8-, and 5-fold, respectively, above binding catalyzed by Hep G2 cells infected with wild-type vaccinia virus. These enzymes, with highest activities, are not known to be expressed in human or rodent lung. CYP2F1 and CYP4B1, two enzymes that are expressed in lung, display only modest 3- and 2-fold respective increased abilities to metabolically activate 4-ipomeanol. Two human forms were inactive and seven other human forms showed activities ranging from 0.5- to 2-fold above control level. Surprisingly, rabbit complementary DNA-expressed CYP4B1 was the most active enzyme (180-fold above control) among all P-450s tested in producing DNA-binding metabolites from this mycotoxin. These studies demonstrate a species difference in 4-ipomeanol metabolism and suggest caution when attempting to extrapolate rodent data to humans.

INTRODUCTION

P-450s² are the principal enzymes involved in oxidative metabolism of numerous drugs, chemical carcinogens, and environmental contaminants. Their substrate specificities can be overlapping among different P-450 forms, can also be form-specific (1, 2), and the expression of P-450 genes varies between species (3). Thus, these characteristics make extrapolation of rodent data on procarcinogen metabolism to humans extremely tenuous and uncertain (2). Due to these difficulties, a direct analysis of the metabolic activities of individual human P-450s is an essential necessity in predicting human response to a given compound.

It has been postulated that the pneumotoxin 4-ipomeanol is activated in the lung by P-450s to electrophilic metabolites responsible for its tissue specific toxicity (4). Based on its preferential metabolism and toxicity in the mammalian lung of several species of laboratory animals, mostly rats and rabbits, a rationale was developed proposing the use of the 4-ipomeanol as a lung cancer chemotherapeutic drug in humans (5). Some human lung cancer cell lines and tumor biopsy specimens are capable of mediating the metabolism of 4-ipomeanol to potentially cytotoxic intermediates. These activities, however, are lower than that expected from rodent studies (6).

Received 2/25/91; accepted 6/14/91.

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² The abbreviations used are: P-450, cytochrome P-450; cDNA, complementary DNA.

Specific enzymes present in human lung that are capable of activating 4-ipomeanol have not been established previously. We therefore studied the ability of 14 P-450s to activate 4-ipomeanol to DNA-binding metabolites using vaccinia virus-mediated cDNA expression of P-450s in human Hep G2 cells and *in situ* binding to cellular DNA. We report that several P-450s can catalyze some activation of 4-ipomeanol and at least three distinct human P-450 forms are capable of high rates of biotransformation of this compound. Striking differences exist in the ability of the rabbit and human CYP4B1 P-450 to metabolically activate 4-ipomeanol.

MATERIALS AND METHODS

The construction and characterization of recombinant vaccinia viruses expressing human CYP proteins CYP1A2 (7), CYP2A6 (8), CYP2B7 (9), CYP2C8 (10), CYP2C9 (10), CYP2F1 (11), CYP3A3 (12), CYP3A4 (13), CYP3A5 (12), and CYP4B1 (14) and mouse CYP1A1 (15) were published. Vaccinia viruses containing the CYP2D6, CYP2E1, and rabbit CYP4B1 were constructed using the same procedure.

[3-¹⁴C]-4-Ipomeanol (specific activity, 25.3 mCi/mmol) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. 2-Amino[9-¹⁴C]fluorene (specific activity, 59.6 mCi/mmol) was purchased from Chemsyn Science Laboratories. Testosterone was obtained from Steraloids, Inc., Wilton, NH.

Confluent cultures of Hep G2 cells, grown as recommended by the American Type Culture Collection, were infected with recombinant vaccinia virus at a multiplicity of infection of 5. Ten μ Ci of [¹⁴C]-4-ipomeanol or 6.25 μ Ci of [¹⁴C]-2-aminofluorene were added to 10 ml of the cell culture medium 12 h after infection. The cells were harvested 24 h after infection and proteins and RNA were digested and removed as described (13). DNA concentrations were determined by absorbance at 260 nm and radioactivity was measured using a liquid scintillation counter. In some cases, testosterone was added at final concentration of 100 nM 1 h before cells were harvested and the amount of 6 β -hydroxytestosterone in the media was evaluated using high performance liquid chromatography (16).

RESULTS

The ability of individual P-450 to activate 4-ipomeanol to DNA-binding metabolites was evaluated by incubating human hepatocellular carcinoma cells infected with P-450 cDNA-containing recombinant vaccinia virus, with [¹⁴C]-4-ipomeanol and measuring 4-ipomeanol metabolites covalently bound to cellular DNA. All P-450s were expressed at similar levels of 15 \pm 3 (SD) pmol/mg total cellular protein in these cells as determined by CO-reduced spectral analysis (13). Significant DNA binding was detected in cells infected with vaccinia viruses expressing human CYP1A2, CYP3A3, and CYP3A4 (Table 1). DNA-binding levels catalyzed by these enzymes are, respectively, 20-, 8-, and 5-fold higher than those obtained in Hep G2 cells infected with wild-type vaccinia virus. CYP2F1 was 3-fold

Table 1 Covalent binding of 4-ipomeanol to cellular DNA in Hep G2 cells^a

Vaccinia virus	Specific binding (dpm/ μ g DNA)
Wild type	0.48 \pm 0.02
CYP1A1 (mouse)	0.98 \pm 0.10 ^b
CYP1A2	9.53 \pm 0.70 ^b
CYP2A3	0.87 \pm 0.06 ^b
CPY2B7	1.67 \pm 0.23 ^b
CYP2C8	1.03 \pm 0.21 ^b
CPY2C9	0.83 \pm 0.06 ^b
CYP2D6	0.57 \pm 0.06
CYP2E1	0.57 \pm 0.15
CYP2F1	1.37 \pm 0.32 ^b
CYP3A3	3.90 \pm 1.57 ^b
CYP3A4	2.33 \pm 0.61 ^b
CYP3A5	0.90 \pm 0.17 ^b
CYP4B1	0.93 \pm 0.25 ^b

^a Mean \pm SD for three experiments.

^b Values are significantly different from control ($P < 0.05$) determined using the Student *t* test.

higher than control whereas all other human P-450s were not significantly active or catalyzed less than 2-fold increased binding. We do not have a vaccinia virus expressing human CYP1A1; however, mouse CYP1A1 did not display much 4-ipomeanol activation activity (Table 1).

Earlier studies demonstrated that purified rabbit CYP4B1 can activate 4-ipomeanol (17). Indeed, rabbit vaccinia virus-expressed CYP4B1 is also capable of activation of 4-ipomeanol at the level 80 times higher than its human orthologous counterpart as measured by the formation of DNA-binding metabolites (Table 2). The specific binding of 4-ipomeanol metabolites demonstrated with rabbit CYP4B1 equals 84 dpm/ μ g DNA which is 180-fold higher than background wild-type vaccinia virus-infected Hep G2 cells and 9-fold higher than that obtained with CYP1A2, the most active human form tested.

To test whether vaccinia virus expressed human CYP4B1 is catalytically active, we examined oxidation of testosterone. Human CYP4B1, despite its inability to activate 4-ipomeanol, was able to catalyze testosterone 6 β -hydroxylation (Table 2). We also examined the metabolism of another mutagen, 2-aminofluorene, and found that this compound is also preferentially activated to DNA-binding metabolites by rabbit CYP4B1 (Table 2). Thus, a marked species difference exist between CYP4B1 in rabbits and humans.

DISCUSSION

Human cytochrome P-450, CYP1A2, CYP2B7, CYP3A3, CYP3A4, and to some extent CYP2F1 are capable of metabolically activating 4-ipomeanol to DNA-binding metabolites in whole cells. The rabbit CYP4B1 form metabolizes this compound and does it with significantly higher catalytic activities than any of the human enzymes as judged by the binding of radiolabeled metabolites to DNA. Mouse CYP1A1, the orthologue of human CYP1A1, only slightly activates 4-ipomeanol. Although we cannot presently rule out that human CYP1A1 activates this toxin, our data are in agreement with the lack of correlation between human CYP1A1 mRNA levels and 4-ipomeanol metabolism in human lung cancer cell lines and normal lung tissue (6).

Lung-specific toxicity of 4-ipomeanol was demonstrated in several mammalian species and covalent binding of activated metabolites to cellular macromolecules was found to be highly correlated with toxicity observed *in vivo* (4, 6, 18). Substantial extrapulmonary covalent binding was also detected *in vivo* in hamster liver and in adult murine kidney. The significance of

covalent and noncovalent interactions in acute cell injury has been reviewed and indeed there is a direct relationship between binding and cell death (19). As summarized in Table 3, activation of 4-ipomeanol to protein-binding metabolites indicates that rabbit lung preparations are 25-fold more active than human lung, lung tumor, and lung tumor-derived cell lines (6, 19, 20). Thus, the large difference between human and rabbit lung in binding of the toxin to cellular macromolecules corroborates our observations made at the level of individual human and rabbit P-450s.

The P-450 enzymes responsible for lung-specific toxicity of 4-ipomeanol were studied in detail only in rabbits where it was found that two enzymes, P450I and P450II, contribute to the formation of electrophilic metabolites of the toxin in a tissue-specific manner (17). The closest respective human counterparts of P450I and P450II are CYP2B7 and CYP4B1, respectively, which in the present study were found not to be capable of significant metabolism of the 4-ipomeanol. Human CYP4B1 activates 4-ipomeanol only at about 1% of the level of the rabbit form of the enzyme. CYP2F1, which is also expressed in human lung, is capable of weakly activating 4-ipomeanol but again at a level markedly lower than the rabbit CYP4B1. Other interspecies differences of CYP4B1 in 2-aminofluorene *N*-hydroxylase and testosterone 6 β -hydroxylase activity are known despite the high 85% similarity in primary amino acid sequence between rabbit and human proteins (14).

4-Ipomeanol has been considered as an agent for lung cancer therapy (5). However, since this compound requires metabolic activation for its cell-killing effect the species differences in P-450s should be carefully considered during development of an appropriate animal model. In addition, the tissue-specific localization of the P-450s and their expression in lung tumor cells should be firmly established. It should be noted that the P-450s examined in this report have not been extensively studied for their expression in lung and other tissues although we believe, based on mRNA measurements, that CYP4B1 and

Table 2 Comparison of covalent binding of 4-ipomeanol and 2-aminofluorene to cellular DNA and formation of 6 β -hydroxytestosterone by rabbit and human forms of CYP4B1 and human CYP1A2^a

Vaccinia virus	Specific binding, dpm/ μ g DNA		6 β -hydroxytestosterone ^b (nmol/flask/h)
	4-Ipomeanol	2-Aminofluorene	
Wild type	0.47 \pm 0.11	0.19 \pm 0.03	ND
CYP4B1 (rabbit)	84.23 \pm 15.84	10.17 \pm 1.68	ND
CYP4B1 (human)	0.98 \pm 0.18	0.30 \pm 0.04	12.91 \pm 2.88
CYP1A2 (human)	8.91 \pm 2.00	9.13 \pm 1.68	ND

^a Means \pm SD for three experiments, other than those in Table 1. ND, not detectable.

^b Testosterone was added to recombinant vaccinia virus-infected Hep G2 cells in 10 ml of culture medium at a final concentration of 100 nM in 25-cm² flasks. The medium was harvested after 1 h and the total nmol of 6 β -hydroxytestosterone produced was measured by high performance liquid chromatography.

Table 3 *In vitro* binding of 4-ipomeanol in rabbit and human tissues

Tissue	4-Ipomeanol binding (pmol/mg protein/min)	Ref.
Rabbit lung, <i>n</i> = 3	500 ^a	19
Human lung, <i>n</i> = 56	18	6
Pulmonary carcinoma, <i>n</i> = 56	18	
Lung cancer cell lines, <i>n</i> = 18	18	
Lung cancer cell lines		
Bronchioloalveolar, <i>n</i> = 2	23	20
Small cell, <i>n</i> = 2	ND	

^a Converted from value reported for microsomal protein fraction. ND, not detectable.

CYP2F1 are among the most abundant lung P-450s. A more thorough investigation of expression of P-450s in human lung awaits sensitive *in situ* determination of P-450 in individual cell types and a comprehensive screening of human lung tumor cells. These studies should provide a solid framework for a decision on application of the toxin to human cancer therapy.

We feel that assignment of specific enzymatic activities to individual cytochrome proteins recently made possible by the use of the vaccinia virus cDNA expression system can contribute to studies of the role of P-450s in cancer drug metabolism and susceptibility of normal lung to toxins and carcinogens. It should be emphasized, however, that whole tissues or other cell types can be biochemically different from the Hep G2 cell model cDNA expression system described herein. For example, lipid compositions and levels of P-450 support enzymes such as the NADPH-P-450 oxidoreductase and cytochrome *b*₅ can vary between different cells. Cellular composition of conjugating enzymes and even conjugating substrates which can trap or detoxify high energy P-450-generated metabolites can effect the toxicity of certain chemicals. Most importantly, levels of individual P-450 forms can vary among the population and even between races. Thus, analysis of P-450 substrate specificities is a meaningful beginning to our understanding of the effects of drugs and environmental chemicals in humans and the development of human enzyme-based systems to analyze human risk assessment (2).

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Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 1991;51:4636-4638.

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