Effects of 8-methoxypsoralen on cytochrome P450 2A13

Linda B. von Weymarn¹, Qing-Yu Zhang², Xinxin Ding² and Paul F. Hollenberg¹,³

¹Department of Pharmacology, University of Michigan, Ann Arbor, Michigan, USA and ²Wadsworth Center, New York State Department of Health, Albany, New York, USA
³To whom correspondence should be addressed
Email: phollen@umich.edu

Cytochrome P450 2A13 efficiently catalyzes the bioactivation of several tobacco-specific nitrosamines in vitro. This efficient bioactivation together with the selective expression of P450 2A13 in the human lung suggests that this P450 may play an important role in the initiation of lung cancer in smokers. Therefore, the identification of potent and selective inhibitors/inactivators of P450 2A13 could potentially help to lower the risk of lung cancer in smokers. In this study, we investigated the ability of 8-methoxypsoralen (8-MOP), a known inhibitor of P450 2A6, to inhibit and inactivate the activities of heterologously expressed P450 2A13 in reconstituted systems. We found that 8-MOP is a potent inhibitor of P450 2A13-mediated metabolism of several compounds, including testosterone, which had not been known to be a P450 2A13 substrate. The Kᵢ for the non-competitive inhibition of P450 2A13-mediated coumarin 7-hydroxylation by 8-MOP was 0.11 μM. The inhibition of P450 2A13 was accompanied by inactivation of the enzyme. Therefore, the observed decrease in activity is most likely due to the inactivation of the enzyme together with competitive or non-competitive inhibition of P450 2A13 by 8-MOP. The inactivation did not result in a loss of native heme, or a significant change in the reduced-Fe⁺ spectrum of the P450, and did not generate any detectable heme adducts. Instead, the inactivation of P450 2A13 by 8-MOP occurred through the formation of an adduct to the apoprotein. LC/MS analysis of the adducted protein indicated an increase in the mass of 232 Da compared with the unadducted protein. This mass shift correlates with the addition of one molecule of 8-MOP plus one atom of oxygen atom to the P450 apoprotein.

Introduction

Enzymes in the cytochrome P450 (P450) super-family of monoxygenases play a crucial role in both the activation and detoxification of many chemical carcinogens. Most P450 enzymes metabolize a number of substrates, and different P450 enzymes often exhibit overlapping substrate specificities. Yet, the rates of metabolism and the stereospecificities differ significantly among different P450s. It has become apparent that a single P450 isoform may play a dominant role in the metabolism of a particular compound in vivo. Therefore, the carcinogenicity or toxicity of a given compound could be determined, at least in part, by the activities of a particular P450 enzyme. Consequently, compounds that effectively inhibit or inactivate this P450 enzyme would be very useful for chemoprevention.

The P450 2A enzymes efficiently metabolize a number of nitrosamine carcinogens in vitro (1–7). These P450s have been implicated as playing significant roles in the metabolic activation of a number of nitrosamines in vivo in both humans and laboratory animals. The three members of the human P450 2A gene family (8) are: P450 2A6, a hepatic coumarin 7-hydroxylase (9), P450 2A13, an extra-hepatic P450 (6) and P450 2A7, which has been reported to be a non-functional enzyme (10). Su et al. recently cloned P450 2A13 from human nasal mucosa and reported that it is a functional enzyme and that it metabolizes several P450 2A6 substrates; however, the metabolic efficiencies for the two enzymes differed significantly (6,11). In addition to being expressed in nasal mucosa, P450 2A13 is also expressed at significant levels in the human lung and trachea. In all three tissues, CYP2A13 was expressed at higher levels than was P450 2A6. Of potential importance for human lung cancer, the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is metabolized to potentially carcinogenic intermediates much more efficiently by P450 2A13 than by any other known human P450 enzyme (6). NNK primarily induces lung tumors in laboratory animals and is thought to play a critical role in the causation of lung cancer in smokers (12). The high rate of NNK metabolism by P450 2A13 together with the relatively high expression of P450 2A13 in the human lung suggests that P450 2A13 could be the primary enzyme in human lung responsible for the metabolic activation of NNK. Therefore, the identification of a potent and specific inhibitor/mechanism-based inactivator of P450 2A13 could lead to approaches to lower the metabolic activation of NNK in the target tissue, and, as a result, potentially lower the risk of lung cancer in smokers.

Mechanism-based inactivation of the P450 enzymes requires P450-mediated metabolism of a substrate to a reactive intermediate that can bind irreversibly to the enzyme, rendering the enzyme inactive. In addition to the aforementioned roles in chemoprevention, mechanism-based inactivators are also useful tools to study the structure–function relationships of individual P450 enzymes. So far, the use of mechanism-based inactivators has aided in determining the orientation of the heme in the P450 active site, and has helped identify portions of the P450 enzymes involved in substrate binding (13–15). Inactivators that bind to the apoprotein are particularly useful for identifying amino acids in the
active site that are important in catalytic activity and for determining the roles of these residues in substrate binding and/or catalysis.

Relatively few studies have focused on the mechanism-based inactivation of the P450 2A enzymes. The only well-characterized inactivator of P450 2A6 is 8-methoxypsoralen (8-MOP), a derivative of the P450 2A6 substrate coumarin (16,17). 8-MOP has been used in the treatment of psoriasis, cutaneous T-cell lymphoma and vitiligo (18–20). 8-MOP was found to inhibit P450 2A6-dependent clearance of nicotine in vivo in human subjects, and to increase the fraction of cigarette smoking-derived NNK that is detoxified through NNAL-glucuronidation, probably as a result of inhibition of hepatic P450 2A6-mediated NNK α-hydroxylation (21). 8-MOP was also found to inhibit NNK-induced lung tumorigenesis in mice, presumably through inhibition of mouse CYP2A5 (22). 8-MOP inactivates P450 2A6 through the formation of a covalent adduct to the apoprotein (16,17); however, the identity of the modified amino acid(s) has not yet been determined. The ability of 8-MOP to act as a mechanism-based inactivator of P450 2A13 has not been investigated. Yet, the high degree of sequence homology between P450 2A6 and P450 2A13 (94% identical), and the overlapping substrate specificities between the two enzymes, made us suspect that 8-MOP might also be a mechanism-based inactivator of P450 2A13.

In this study we obtained P450 2A13 through heterologous expression in Escherichia coli. The P450 2A13 generated in this system was partially purified. The purified P450, as well as P450 2A13 expressed in insect Sf9 cell microsomes (6), was used for determination of the effects of 8-MOP on P450 2A13 activity, with use of a number of different substrates. Here we show that 8-MOP is a potent inhibitor as well as an inactivator of P450 2A13. We also present the results of our initial characterization of the mechanism of inactivation of P450 2A13 by 8-MOP.

Materials and methods

Materials

Dilauroyl-t-α-phosphatidylcholine (DLPC), NADPH, bovine serum albumin (BSA), coumarin, hexamethylenephosphoramide (HMPA), 7-hydroxycoumarin (7-OHC), 8-MOP and catalase were purchased from Sigma Chemical (St Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was purchased from Molecular Probes (Eugene, OR). 7-Hydroxy-4-(trifluoromethyl) coumarin (7-HFC) was purchased from Oakwood Products (West Columbia, SC). Testosterone, 15α-, 6β- and 16α-hydroxytestosterone were purchased from Steraloids (Newport, RI). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL).

Expression and purification of P450 2A13

The P450 2A13 cDNA that was used previously for baculoviral expression (6) was cloned into the pLW01 vector expression for expression in E.coli DH5α cells. The second codon was changed from leucine to alanine for facilitation in E.coli. The primers used for cloning and mutagenesis were: 5′-ccagcagctgctcgacgctgtgctgctgctg-3′ (forward primer; start codon marked in bold, mutated bases are underlined) and 5′-ccacagcagctgctcgacgctgtgctgctg-3′ (reverse primer; complementary to the multiple cloning site of the P450 2A13 cDNA plasmid). The integrity of the P450 2A13 sequence after cloning into pLW01 was confirmed by sequencing.

Expression of P450 2A13 in DH5α cells was accomplished using essentially the same protocol that was used for the expression of P450 2A6 and P450 3A4 (23,24). The pLW01 plasmid containing P450 2A13 was transformed into DH5α cells. Cells were grown in 10 ml LB media containing 100 μg/ml ampicillin at 37°C overnight prior to expanding into 1 l TB peptone containing ampicillin (100 mg/ml), 1 mM thiamine and trace elements. The cells were grown at 32°C until the OD at 600 nm reached 0.6 at which time α-aminolevulinic acid (0.5 mM) was added. The cells were induced with 1 mM isopropyl-β-D-thiogalactoside when the OD at 600 was ~1.2 and then allowed to grow for 48 h at 32°C. The cells were harvested and purified according to published protocols except only a DEAE-Sepharose column and a SP-Sephadex column were used for the purification (25). The purity of the enzyme was determined by LC/MS.

Catalytic activity of purified P450 2A13

P450 2A13 was reconstituted with NADPH-P450 oxidoreductase (reductase) and lipid (DLPC) for 45 min at 4°C. The reductase used was expressed in E.coli Topp 3 cells and purified according to a published protocol (26). After the reconstitution, catalase and Tris buffer were added to the reconstituted enzymes to give an incubation mixture containing 1 pmol/μl P450 2A13, 2 pmol/μl reductase, 0.1 μg/μl lipid, 26 μg/μl catalase and 50 mM Tris buffer, pH 7.4. The molar ratio of P450 to reductase was 1:2 unless otherwise noted. All the times used in the kinetics experiments were within the linear range of the product formation by the enzyme and the substrate concentrations were optimized.

Cumarin 7-hydroxylation

The formation of 7-OHC was measured according to a previously published protocol (4). Aliquots of the reconstituted enzyme solution (containing 10 pmol P450 2A13) were added to reaction mixtures containing coumarin (0.1–20 μM) and NADPH (1 mM) in 50 mM Tris buffer, pH 7.4. The final reaction volume was 300 μl. The reaction was allowed to proceed for 10 min at either 30 or 37°C prior to termination of the reaction by the addition of 20 μl of 15% trichloroacetic acid (TCA). The 7-OHC formed was analyzed using HPLC system 1 (below) with fluorescence detection (excitation wavelength, 350 nm; emission wavelength, 453 nm). Quantification was achieved by comparison with a standard curve of pmol 7-OHC versus peak area. The ability of 8-MOP to inhibit P450 2A13-mediated coumarin 7-hydroxylation was measured with 0.1–1.0 μM 8-MOP in the reaction mixtures.

Testosterone metabolism

The ability of P450 2A13 to metabolize testosterone was determined as described previously (3). Aliquots of the reconstituted enzyme solution (containing 25 or 50 pmol P450 2A13) were added to reaction mixtures containing testosterone (1–200 μM) and NADPH (1 mM) in 50 mM HEPES buffer, pH 7.4, containing 40 μg/ml BSA and 1 mM MgCl2. The total reaction volume was 1.0 ml. The samples were incubated for 20 min at 30°C following the addition of the reconstituted enzyme solution. The reactions were terminated by the addition of 1 ml ethyl acetate. The metabolites were extracted twice, each time with a 1 ml aliquot of ethyl acetate, dried under N2 gas, and were re-suspended in methanol. The testosterone metabolites were analyzed by reversed-phase HPLC (system II) with UV detection (at 254 nm), and the formation of 15α-hydroxytestosterone was quantified by comparison with an internal standard (6β-hydroxytestosterone).

7-EFC O-deethylation

Aliquots of the reconstituted enzyme solution (containing 25 pmol P450 2A13) were added to reaction mixtures containing (1–100 μM) 7-EFC, 1 mM NADPH and 40 μg/ml BSA in 50 mM Tris buffer, pH 7.4. The total reaction volume was 1.0 ml. The samples were incubated for 20 min at 30°C following the addition of the reconstituted enzyme solution. The reaction was quenched by the addition of 334 μl acetonitrile. The 7-EFC O-deethylation activity was measured as described previously (27). The conversion of 7-EFC to 7-HFC was measured on a SLM-Aminco model SPF-500C spectrophotometer (Thermo Spectronic, Rochester, NY) at room temperature with an excitation wavelength of 410 nm and an emission wavelength of 510 nm. Quantification was achieved by comparison with a standard curve of pmol 7-HFC versus relative fluorescence intensity.

Effect of 8-MOP on purified P450 2A13-mediated metabolism

P450 2A13 was reconstituted with reductase and lipid for 45 min at 4°C. The primary reaction mixture contained 1 pmol/μl P450, 2 pmol/μl reductase, 0.1 μg/μl lipid, 26 μg/μl catalase, 8-MOP (0–10 μM) and 1 mM NADPH in 50 mM Tris buffer, pH 7.4. The primary reaction mixture was incubated for 5 min at 30°C prior to the addition of NADPH. The inactivation experiments were run at 30°C to minimize losses in activity in the control samples (~8-MOP, +NADPH), probably due to the formation of peroxide through a futile redox cycle that was observed when the samples were incubated at 37°C. Following addition of NADPH, aliquots were removed from the primary reaction at the indicated time points and the amount of catalytic activity remaining, the amount of spectrally detectable P450 remaining, and the amount of native heme remaining was determined as described below to aid in determining the extent of inactivation as well as the mechanism of inactivation. Part of each sample was also put through a G50 Sephadex spin-column.
to remove small molecules such as 8-MOP and NADPH to eliminate the inhibition of P450 2A13 by 8-MOP.

**Effect of 8-MOP on P450 2A13-mediated testosterone metabolism**

At 0 and 5 min following NADPH addition, aliquots of the primary reaction mixture containing 8-MOP (0 or 6 μM) were added to a secondary reaction mixture containing testosterone (200 μM) and NADPH (1 mM) in a 50 mM HEPES buffer, pH 7.4, containing 40 μg/ml BSA and 1 mM MgCl2 (in a final volume of 1.0 ml). The loss in testosterone metabolism was determined as described above. Due to the high degree of competitive inhibition by 8-MOP, it was necessary to pass an aliquot of each sample (containing 250 pmol P450) through a G5 Sephadex spin-column to remove small molecules such as 8-MOP and NADPH. The flow-through from the spin-column was assayed for loss of testosterone activity as well as in reduced CO spectrum and native heme (see below).

**Spectral measurements of the reduced CO complex**

P450 2A13, reductase and lipid were reconstituted and the primary reaction was prepared as described above. The primary reaction mixture contained 6 μM 8-MOP. After a 5-min incubation at 30°C in the presence (inactive sample) or absence (control sample) of NADPH, 100 μM (100 pmol P450) of the primary reaction mixture was added to 900 μl of an ice-cold quench buffer containing 40% glycerol and 0.6% Nonidet P-40 in 50 mM potassium phosphate (pH 7.4). The reduced CO spectrum was measured on a DW2 UV/VIS spectrophotometer (SLM Aminco, Urbana, IL) with an OLIS spectroscopy controller, Waters 501 series pumps, a Waters 996 photodiode-array detector, and a Waters 717 autosampler (Waters, Milford, MA); the same system was used for HPLC Systems III and IV. The mobile phase consisted of water (A) and acetonitrile (B). The elution was accomplished by holding the mobile phase at 70% A:30% B for 5 min followed by a linear gradient to 80% B in 25 min and then to 95% B in 5 min. The elution of heme and heme related peaks was monitored at 405 nm. The flow rate was 1 ml/min.

**HPLC System IV.** The samples were injected onto an Agilent Zorbax 300SB-C3 reversed phase HPLC column that was equilibrated with 60% water containing 0.1% TFA (A) and 40% acetonitrile containing 0.1% TFA (B). The protein components were eluted by maintaining the initial concentrations of the mobile phase at 60% A:40% B for 5 min followed by a linear gradient to 80% B in 25 min and then to 90% B in 5 min, followed by holding at 90% B for 15 min. The flow rate was 0.3 ml/min.

**Kinetic analysis**

Km, Vmax, and Kc values were determined using the Ez-Fit 5 kinetics program from Perrella Scientific (Amherst, NH). This program uses a non-linear regression method of curve fitting and the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence.

**Results**

P450 2A13 was heterologously expressed in E.coli for the first time, and was purified from bacterial membranes for metabolic studies. The expression and purification protocols routinely yielded 10-30 nmol of purified P450 2A13 per liter of cells in culture. The P450 content of the purified fraction used in this study, as measured by the reduced CO spectrum, was 1.5 nmol/mg protein. The enzyme was not purified to homogeneity; according to LC/MS analysis one significant protein impurity was present in the P450 2A13 solution (data not shown).

The ability of P450 2A13 in the reconstituted system to catalyze coumarin 7-hydroxylation was determined. Several P450 2A enzymes efficiently and exclusively metabolize coumarin at the 7-position; therefore, coumarin 7-hydroxylation is often used as a probe activity for the P450 2A enzymes. The kinetics of P450 2A13-mediated coumarin 7-hydroxylation were determined at two different temperatures, 30 and 37°C. The reactions followed Michaelis-Menten kinetics at both temperatures. The graph for the kinetics study at 37°C is shown in Figure 1. As shown in Table I, the Km and Vmax at 30°C were 0.7 μM and 0.29 nmol/min/nmol P450, respectively. As expected, no significant change in the Km (1.1 μM) was observed upon increasing the temperature to 37°C but the Vmax increased to 0.39 nmol/min/nmol P450 (Figure 1 and Table I). The ability of P450 2A13 to catalyze 7-EFC O-deethylations and testosterone hydroxylation was also determined. The P450 2A13-mediated 7-EFC O-deethylation followed Michaelis-Menten kinetics, with Km and Vmax values of 1.2 μM and 0.14 nmol/min/nmol P450, respectively (Table I). A number of P450 2A enzymes can efficiently catalyze testosterone 16α-hydroxylation; however, the ability of P450 2A13 to catalyze this reaction has not been reported previously. As shown in Figure 2, P450 2A13 hydroxylates testosterone...
primarily at the 15α position. In addition to the 15α-hydroxytestosterone, a trace amount of 6β-hydroxytestosterone was formed. The testosterone 15α-hydroxylation by reconstituted P450 2A13 followed Michaelis–Menton kinetics with a $K_m$ of 13 $\mu$M and a $V_{max}$ of 1.7 nmol/min/nmol P450 (Table I).

The effect of 8-MOP on P450 2A13-mediated coumarin 7-hydroxylation was investigated. 8-MOP was a potent inhibitor of P450 2A13 (Figure 3). The inhibition of P450 2A13 by 8-MOP had a $K_I$ of 0.11 $\pm$ 0.03 $\mu$M and the inhibition resulted in a change in $V_{max}$ with the $K_m$ staying constant (1.4, 1.8 and 1.4 $\mu$M, respectively for the 0.1, 0.5 and 1.0 $\mu$M 8-MOP concentrations) suggesting non-competitive inhibition (Figure 3). The low $K_I$ for the inhibition of P450 2A13-mediated coumarin 7-hydroxylation by 8-MOP would likely mask any mechanism-based inactivation of P450 2A13 by 8-MOP. Therefore, a G50 Sephadex spin-column was used to remove small molecules, such as 8-MOP, after pre-incubation of the reconstituted P450 2A13 with 8-MOP in the presence or absence of NADPH in order to determine whether 8-MOP could act as a mechanism-based inactivator of P450 2A13. The loss in the coumarin 7-hydroxylation activity was not significantly different in the presence (15%) or absence (21%) of NADPH in samples, which were not applied to the

Table I. Kinetic parameters for coumarin, 7-EFC and testosterone metabolism by reconstituted P450 2A13*

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ (nmol/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin 7-hydroxylation, 30°C</td>
<td>0.7 ± 0.08</td>
<td>0.29 ± 0.009</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation, 37°C</td>
<td>1.1 ± 0.2</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>7-EFC O-deethylation</td>
<td>1.2 ± 0.2</td>
<td>0.14 ± 0.004</td>
</tr>
<tr>
<td>Testosterone 15α-hydroxylation</td>
<td>13 ± 3</td>
<td>1.7 ± 0.11</td>
</tr>
</tbody>
</table>

*Experimental details are described in the Materials and methods. Coumarin (0–20 $\mu$M), 7-EFC (0–200 $\mu$M) or testosterone (0–200 $\mu$M) was incubated with reconstituted P450 2A13 at 30 or 37°C for 10 min (coumarin), at 30°C for 20 min (7-EFC) or at 30°C for 30 min (testosterone). The kinetic parameters were determined using the Ez-Fit 5 kinetics software. Each value is the average of three independent experiments performed in duplicate.

![Fig. 1. Kinetics of P450 2A13-mediated coumarin 7-hydroxylation.](image1)

Reconstituted P450 2A13 was incubated with coumarin (0.1, 0.2, 0.4, 0.8, 1, 2, 5, 10 and 20 $\mu$M) and NADPH for 10 min at 37°C as described in the Materials and methods. The formation of 7-OHC was determined by reverse-phase HPLC with fluorescence detection as described in the Materials and methods. The data shown represent the mean and standard deviations from three experiments done in duplicate. The inset is a Lineweaver–Burk plot of the kinetic data.

![Fig. 2. HPLC analysis of P450 2A13-mediated testosterone metabolism.](image2)

(A) Elution of the authentic standards 15α-hydroxytestosterone (15α-OH), 6β-hydroxytestosterone (6β-OH), 16α-hydroxytestosterone (16α-OH) and testosterone. (B) Testosterone (200 $\mu$M) was incubated for 20 min with 50 pmol P450 2A13 in the reconstituted system as described in the Materials and methods. The products were measured at 254 nm.

![Fig. 3. Inhibition of P450 2A13-mediated coumarin 7-hydroxylation by 8-MOP.](image3)

The inhibition of P450 2A13-mediated coumarin 7-hydroxylation was determined as described in the Materials and methods. 8-MOP was added at concentrations of 0 $\mu$M (filled square), 0.1 $\mu$M (filled triangle), 0.5 $\mu$M (inverted filled triangle) and 1.0 $\mu$M (filled diamond) to samples containing varying amounts of coumarin as indicated (0.4–20 $\mu$M). The formation of 7-OHC was determined by HPLC analysis with fluorescence detection. Curves were generated using non-linear regression analysis. The data shown represent the mean and standard deviations from three experiments done in duplicate.
Table II. Effect of 8-MOP on P450 2A13-mediated testosterone 15α-hydroxylation a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>8-MOP (μM)</th>
<th>Rate of product formation (nmol/min/nmol P450)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>0</td>
<td>0.18 ± 0.006</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.09 ± 0.013</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.05 ± 0.008</td>
<td>82</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0</td>
<td>1.4 ± 0.27</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.3 ± 0.07</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.2 ± 0.03</td>
<td>89</td>
</tr>
<tr>
<td>HMPA</td>
<td>0</td>
<td>22.8 ± 1.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>17.8 ± 1.0</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.2 ± 0.2</td>
<td>95</td>
</tr>
</tbody>
</table>

*Samples were prepared as described in the Materials and methods. Microsome samples and those containing 8-MOP (6 μM) were incubated for 5 min at 30°C in the presence or absence of NADPH. The testosterone 15α-hydroxylase activity was measured before and after running the samples through a spin-column to remove small molecules such as 8-MOP. All samples were run in duplicate on three separate occasions. % Activity remaining was calculated based on control samples (8-MOP, NADPH). &

Table III. Inhibition/inactivation of coumarin, testosterone and HMPA metabolism in microsomes containing baculovirus expressed P450 2A13 by 8-MOP a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>8-MOP (μM)</th>
<th>Rate of product formation (nmol/min/nmol P450)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>0</td>
<td>0.18 ± 0.006</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.09 ± 0.013</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.05 ± 0.008</td>
<td>82</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0</td>
<td>1.4 ± 0.27</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.3 ± 0.07</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.2 ± 0.03</td>
<td>89</td>
</tr>
<tr>
<td>HMPA</td>
<td>0</td>
<td>22.8 ± 1.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>17.8 ± 1.0</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.2 ± 0.2</td>
<td>95</td>
</tr>
</tbody>
</table>

*Samples were prepared as described in the Materials and methods. Microsomes containing P450 2A13 (5 pmol for coumarin and testosterone assays, 50 pmol for HMPA assays) were incubated with coumarin (20 μM), testosterone (200 μM) or HMPA (1 mM) in the presence or absence of 8-MOP. The rate of metabolism was determined in duplicate on three separate occasions.

The lipid environment can have a significant effect on P450-catalyzed metabolism (31,32). Therefore, the effect of 8-MOP on heterologously expressed P450 2A13 contained in SF9 microsomes was determined using three different substrates, coumarin, testosterone and HMPA (Table III). The loss in coumarin 7-hydroxylation at 0.5 μM 8-MOP was 49%. At 1 μM there was almost a complete loss in activity (82%) (Table III). Exposing the microsomes to 1 μM 8-MOP also resulted in an almost complete loss in HMPA N-demethylation, 86%. Similar results were observed with testosterone as a substrate; although, the testosterone metabolism was almost completely inhibited at 0.5 μM 8-MOP and no significant additional loss in activity was observed at 1.0 μM 8-MOP (Table III).

The mechanism of the inactivation of P450 2A13 by 8-MOP was investigated. The loss in activity after elution from the spin-column was not accompanied by a loss in native heme as measured by HPLC (Table IV), and no heme adducts were observed by HPLC analysis monitoring at 405 nm (data not shown). A small loss (20%) in the reduced CO spectra was observed both before and after elution from the spin-column even though there was no heme loss (Table IV). The discrepancy between the loss in activity and the loss in native heme and reduced CO spectra suggested the formation of adducts to the apo-protein as the main mechanism for the inactivation of P450 2A13 by 8-MOP.

The ability of 8-MOP to generate covalent adducts to the apo-protein of P450 2A13 was analyzed using ESI-LC/MS. The HPLC method used was able to separate catalase, proteolytically clipped ‘short’ reductase, active reductase and P450 2A13 (Figure 4, top). The protein envelope for P450 2A13 is shown in the inset of Figure 4 (top). The resulting spectra of inactive (+8-MOP, +NADPH) and exposed control (+8-MOP, –NADPH) samples after deconvolution of the P450 2A13-containing protein envelope demonstrate that the inactivated sample contained two different protein species with a mass difference of 232 mass units (Figure 4, bottom). The peak with a mass of 56656 mass units corresponds to native P450 2A13 (theoretical mass = 56645) and is present in both the exposed control (inset) and the inactive sample (Figure 4, bottom). A mass difference of 232 mass units is equivalent to the addition of one molecule of 8-MOP with one oxygen atom attached. The amount of adduct formed was dependent upon the concentration of 8-MOP and the inactivation time (data not shown).

Discussion

P450 2A13 has recently received increased attention due to its ability to catalyze the efficient metabolic activation of NNK, a tobacco-specific lung carcinogen (6,11,33–36). The rapid rate of carcinogen activation together with the relatively high level of expression of P450 2A13 in the human lung suggests that P450 2A13 might play a role in the initiation of lung cancer in smokers. Therefore, efforts to identify effective P450 2A13 inhibitors or inactivators are warranted. The availability of purified P450 2A13 should aid in gaining a better understanding of the mechanisms of carcinogen activation by this
enzyme. Although P450 2A13 was expressed previously using the baculoviral system (6), the same enzyme was generated using a bacterial expression system in the present study to allow more rapid production and purification for metabolic studies. A previous study has shown that converting codon two of P450 2A6 to an alanine yields an increase in expression in E.coli compared with the expression of the wild-type P450 2A6 (24). Therefore, the leucine at codon two of P450 2A13 was mutated to an alanine. The yield of pure enzyme was in the range of 10–30 nmol/l of cells, which is almost 10-fold lower than the reported expression of P450 2A6 using a similar construct and method of expression (24).

Coumarin is often used as a biomarker for the P450 2A enzymes in vivo and in vitro due to the high rate and enzyme-selectivity of coumarin 7-hydroxylation by these enzymes (37–39). Microsomes containing baculovirus expressed P450 2A13 has been shown to efficiently catalyze coumarin 7-hydroxylation with a $K_m$ of 0.48 μM and a $V_{max}$ of 0.15 nmol/min/nmol P450 (11). We have demonstrated here that reconstituted purified P450 2A13 has a $K_m$ (0.7–1.1 μM depending on temperature) similar to that reported in microsomes; however, the $V_{max}$ reported here (Table I) is higher (0.39 nmol/min/nmol P450) than what has been reported in microsomes (0.15 nmol/min/nmol P450). There are several possible reasons for the difference in $V_{max}$: (i) different lipid compositions have been shown to have different effects on P450-catalyzed metabolic rates (31,32), in this case we were using pure DLPC in our reconstitution compared with the mixture of lipids that are present in the microsomes from Sf9 insect cells; (ii) the expressed and purified reductase used in the experiments described here was based on the rat sequence whereas the reductase used previously was cloned from humans; and (iii) there could be a slight difference in the folding of the enzyme in E.coli compared with Sf9 cells.

We have also shown for the first time that P450 2A13 is an efficient testosterone 15α-hydroxylase with a $K_m$ of 13 μM and a $V_{max}$ of 1.7 nmol/min/nmol P450 (Figure 2 and Table I). Several other P450 2A enzymes, such as rat 2A3, mouse P450 2A4, rabbit P450 2A11 and hamster P450 2A14 have been reported to catalyze the 15α-hydroxylation of testosterone; however, P450 2A6 does not catalyze this reaction (1,40–43). This is the first substrate tested that is a substrate of P450 2A13 but not of P450 2A6. This novel discovery could potentially be used as an effective tool in vivo to differentiate between these highly homologous enzymes where inhibitory antibodies have failed to distinguish between the two enzymes.

The most potent inactivator of a P450 2A enzyme reported in the literature is 8-MOP, which inactivates P450 2A6 in a time-, concentration- and NADPH-dependent manner through the formation of an adduct to the apo-protein (16,17). We have here shown that 8-MOP is a potent inhibitor/inactivator of P450 2A13 as well as P450 2A6. The $K_i$ for the inhibition of P450 2A13-mediated coumarin 7-hydroxylation was 0.11 μM (Figure 2), which is very similar to the $K_i$ values reported for the inhibition of P450 2A6-mediated coumarin 7-hydroxylation by 8-MOP in vitro and in vivo (16,17,44,45). The inhibition of P450 2A13 by 8-MOP decreases the $V_{max}$ while keeping the $K_m$ constant, suggesting that the inhibition is non-competitive (Figure 2). The non-competitive mechanism of inhibition could, among other things, suggest that the inhibition of P450 2A13 by 8-MOP is accompanied by inactivation of the enzyme. Mechanism-based inactivation would decrease the $V_{max}$ by killing a fraction of the enzyme, while the enzyme molecules not yet inactivated would still exhibit the same $K_m$.

The effect of 8-MOP on microsomes containing baculovirus-expressed P450 2A13 was also determined as a comparison with the results obtained with the purified P450 2A13 that was produced using a bacterial expression system. It has been suggested that the expression of P450 enzymes in Sf9 insect cells provides a more mammalian-like expression environment, which could have an influence on the catalytic activity and/or the specificity of the enzyme. In addition, the lipid composition of baculovirus microsomes is different from that of either E.coli or mammalian cells. When comparing the different lipid environments (baculovirus microsomes versus enzyme reconstituted with DLPC), keeping the P450:reductase ratios and incubation conditions identical, the rate of coumarin 7-hydroxylation was lower for the microsomes containing baculovirus expressed P450 2A13 than for the purified reconstituted P450 2A13 expressed in E.coli cells, 0.18 compared with 0.39 nmol/min/nmol P450, respectively. Surprisingly, the rate of coumarin 7-hydroxylation at 20 μM coumarin reported here for the microsomes containing baculovirus expressed P450 2A13 was almost 2.5-fold higher than the previously reported $V_{max}$ for the same baculovirus expressed P450.

**Fig. 4.** ESI-LC/MS analysis of adduct formation to the P450 2A13 apo-protein upon inactivation by 8-MOP. Reconstituted P450 2A13 was incubated with 8-MOP (6 μM) in the presence (inactive sample) or absence (exposed control) of NADPH (1 mM) as described in the Materials and methods. (Top) Total ion chromatogram of an inactivated sample with the protein envelope for P450 2A13 shown in the inset. (Bottom) Deconvoluted spectrum of the P450 peak from an inactivated sample. The corresponding deconvoluted spectrum of an exposed control sample is shown in the inset.
the rate of HMPA metabolism by the baculovirus expressed P450 2A13 was also higher than previously reported (22.8 versus 4.8 nmol/min/nmol P450) (46). The discrepancy between the values reported here and the previously published rates could be due to the use of rat reductase, instead of rabbit or human reductase, used in this study. Data from our lab suggests that rat reductase can support higher human P450 2A activity than rabbit reductase (X.Ding, unpublished results). Changing the lipid environment of P450 2A13 from reconstitution with just DLPC to the more complex lipid environment encountered in Sf9 microsomes altered the rate of P450-mediated coumarin metabolism. However, no conclusions can be drawn regarding the activity of P450 2A13 in vivo since neither system mimics the lipid environment in human lung cells.

The ability of 8-MOP to inhibit the microsomes containing baculovirus expressed P450 2A13 did not differ significantly among the substrates (the substrate concentration was at least 10 times the $K_m$ for each substrate). At 1 $\mu$M 8-MOP the ability of P450 2A13 to metabolize coumarin, testosterone and HMPA was almost completely abolished. The inhibition of coumarin 7-hydroxylation by 0.5 and 1 $\mu$M 8-MOP did not differ significantly between purified enzyme (60% and 86% inhibition) and the baculovirus expressed microsomes (49 and 82% inhibition). This suggests that even though the lipid environment does not affect coumarin 7-hydroxylation it does not affect the inhibition of P450 2A13 by 8-MOP.

We have shown here that 8-MOP is both a potent inhibitor and a mechanism-based inactivator of P450 2A13. Determining whether 8-MOP is a mechanism-based inactivator of P450 2A13 was difficult due to the potent inhibition of the P450 2A13 catalytic activity by 8-MOP. The loss in P450 2A13-mediated testosterone 15α-hydroxylation activity after preincubation in the presence of 8-MOP was the same in the inactive sample (+8-MOP, +NADPH) as it was in the exposed control sample (+8-MOP, -NADPH) (Table II). There was an 80% loss of testosterone 15α-hydroxylation activity in the exposed control, even though the concentration of 8-MOP in the secondary reaction was diluted from 6 $\mu$M in the primary reaction to only 0.15 $\mu$M (Table II). The loss of activity in the exposed control has to be due primarily to inhibition of the enzyme since no NADPH is present in the primary reaction to facilitate catalytic turnover, an absolute requirement for mechanism-based inactivation. Reducing the concentration of 8-MOP enough to completely avoid inhibition in the secondary reaction resulted in no significant inactivation of the enzyme (data not shown). The removal of small molecules such as 8-MOP and NADPH by running the samples through a spin-column restored the activity of the exposed control sample to 100%, whereas the inactivated sample still exhibited a 60% loss in activity. This suggests that a portion of the activity loss at 6 $\mu$M 8-MOP is due to mechanism-based inactivation and not inhibition, which is supported by the non-competitive mechanism of inhibition that was observed. The loss in activity was not accompanied by a loss in native heme and only a slight loss in the reduced CO spectrum was observed. Running the samples through spin-columns did not affect the heme loss or the reduced CO spectrum. These results suggest that the mechanism of inactivation of P450 2A13, like P450 2A6, is through the formation of an adduct to the apo-protein (16,17). The presence of adducted apo-protein was shown by ESI-LC/MS analysis. An adduct to the P450 2A13 apo-protein corresponding to one 8-MOP molecule and one oxygen atom was observed in samples inactivated by 8-MOP. The adduct was not observed in exposed control samples indicating that the formation of the adduct is dependent on both 8-MOP and NADPH. No adducts were observed on either reductase or catalase indicating that the formation of the adduct is specific to P450 2A13 (data not shown). These results differ from those of Koenigs et al., who reported significant binding of 8-MOP to both P450 2A6 and reductase when using the purified reconstituted enzyme (16). The amount of adduct formed, as determined by whole protein LC/MS analysis, is dependent on the amount of 8-MOP in the incubation as well as the incubation time (data not shown).

The formation of adducts to the apo-protein is potentially of great importance for investigating the structure-function relationships of P450 2A13. Identifying the amino acid residue(s) that are modified in the active site could give insights into the active site topology of P450 2A13, and comparisons between the sites of modification on P450 2A13 and 2A6 could give insights into what features are important for the differences observed in the rates of NNK metabolism by these two enzymes. Due to the efficient metabolic activation of the tobacco-specific nitrosamines NNK and the expression of P450 2A13 in human lung tissue it is important to better understand the factors governing catalytic activity. The critical structural features of P450 2A13 that determine the efficient metabolic activation of NNK by P450 2A13 have not yet been explored.

Finding a potent and specific inhibitor/inactivator of P450 2A13 could ultimately lead to approaches for lowering the risk of lung cancer in smokers due to NNK exposure. The effects of 8-MOP on a number of other human P450 enzymes including P450s 1A2, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 have been reported previously (17,45,47). Of these P450s, only 1A2 and 2B6, in addition to the P450 2As, have been reported to be inhibited by low concentrations of 8-MOP ($K_i$ < 5 $\mu$M) (17,47). Although several other P450 enzymes have been reported to be inhibited by 8-MOP, the reported $K_i$’s are significantly higher than that for P450 2A13. Furthermore, the inhibition seemed to be much more potent in studies with purified enzymes compared with human liver microsome studies (17,47,48). The potent and relatively specific inhibition/inactivation of both functional human P450 2A enzymes, 2A6 and 2A13, by 8-MOP makes 8-MOP a good candidate for the development of a drug that can lower the risk of lung cancer in smokers. Inhibition of hepatic P450 2A6 by 8-MOP, has been shown to increase plasma nicotine levels and decrease nicotine clearance and metabolism in humans in vivo. Therefore, it could potentially decrease tobacco exposure in addicted smokers by reducing the number of cigarettes smoked in order to maintain a steady nicotine level hence decreasing the exposure to tobacco-specific carcinogens (21). In addition, inhibition of respiratory tract P450 2A13 could block target tissue metabolic activation of 2A13 substrates, including tobacco-specific nitrosamines. However, there are several reasons why 8-MOP would not be a good chemopreventive agent. Animal studies have shown that the effect of 8-MOP on P450 enzymes in vivo is biphasic (49). The initial inhibition of P450 activity is followed by an induction of the levels of P450 enzyme in the rat liver. However, 8-MOP did not result in an up-regulation of all the P450 enzymes studied, and the effect of 8-MOP on the P450 2A enzymes was not investigated. If P450 2A6 and 2A13 were up-regulated in vivo in humans, the long-term effects of 8-MOP in smokers might be an increase in lung cancer risk rather than a decrease. An
increase in lung cancer risk could in this case be due to the increased bioactivation of NNK and other tobacco-specific nitrosamines by P450 2A13, as well as an increased smoking behavior due to the increased metabolism of nicotine by the up-regulated P450 2A6. In addition, there is some evidence that 8-MOP is carcinogenic in male F344/N rats (50). Therefore, chronic administration of 8-MOP to smokers might not be beneficial. However, 8-MOP can still be used as a probe to further our understanding of the structure–function relationships within the P450 2A subfamily. Structural information on the active site structure of the human P450 2A enzymes might lead to the development of an 8-MOP analog that has all the beneficial properties of 8-MOP, i.e. very high inhibition/inactivation and high specificity, without the negative properties.

Acknowledgements

The research described in this article was supported in part by a postdoctoral fellowship to L.B.v.W. from Philip Morris USA. The work was also supported in part by research grants CA 092596 (to X.D.) and CA 16954 (to P.F.H.) from the National Institutes of Health.

References

Effects of 8-MOP on P450 2A13

Received September 10, 2004; revised November 11, 2004; accepted November 21, 2004