Short Communication

COMPARATIVE METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINES 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE AND 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANOL BY RAT CYTOCHROME P450 2A3 AND HUMAN CYTOCHROME P450 2A13

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ABSTRACT:

The tobacco-specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) and its carbonyl-reduction product, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol (NNAL), are potent lung carcinogens in rats and are presumed human lung carcinogens. NNK and NNAL are bioactivated to DNA-binding intermediates by cytochrome P450s (P450s) (Hecht, 1998; Ding et al., 2000). Therefore, it is important to determine which P450s are efficient catalysts of this metabolic transformation. In this study, the kinetic parameters for NNK and NNAL metabolism were determined for two extrahepatic P450s that are expressed in the lung: rat P450 2A3 and human P450 2A13. P450s 2A3 and 2A13 exhibited V_max values for NNK 4-hydroxylation of 10.8 ± 0.4 and 13.8 ± 0.8 pmol min⁻¹ pmol P450⁻¹, respectively; the corresponding K_m values were 4.6 ± 0.5 and 3.6 ± 0.7 μM. The respective V_max values for P450 2A3- and 2A13-mediated N-methyl hydroxylation of NNK were 8.2 ± 0.3 and 4.6 ± 0.2 pmol min⁻¹ pmol P450⁻¹. These data indicate that P450s 2A3 and 2A13 are both efficient catalysts of the metabolic activation of NNK and are, along with mouse P450 2A5, the best catalysts of this reaction currently known. Both enzymes also catalyzed the α-hydroxylation and N-oxidation of NNAL, and its oxidation to NNK. In general, V_max/K_m values for NNAL metabolism were 1 to 2 orders of magnitude lower than those for NNK metabolism, and P450 2A3 was a slightly better catalyst of NNAL metabolism than was P450 2A13. Given the exquisite sensitivity of the rat lung to NNK-induced carcinogenesis, the efficient bioactivation of NNK by rat P450 2A3, and the similar catalytic efficiency of P450s 2A3 and 2A13, P450 2A13 may be an important contributor to NNK bioactivation in the human lung.

The tobacco-specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK)¹ and its carbonyl-reduction product, NNAL, are potent and selective inducers of adenocarcinoma in the rat lung, and are thought to play a role in human lung cancer associated with tobacco use (Hecht, 1998). The sensitivity of the rat lung to NNK- and NNAL-induced carcinogenesis is likely due, at least in part, to tissue-specific conversion of these carcinogens to electrophilic DNA-binding intermediates by cytochrome P450s (P450s) (Hecht, 1998; Ding and Kaminski, 2003). Therefore, it is important to determine which P450s are responsible for NNK and NNAL bioactivation in animal models and humans. Both NNK and NNAL are bioactivated via P450-mediated hydroxylation of the carbon atoms adjacent to the nitroso moiety (i.e., α-hydroxylation), as depicted in Fig. 1 (Hecht, 1998). Hydroxylation at the α-methylene position ultimately generates OPB and lactol from NNK and NNAL, respectively, and generates a DNA-methylating agent (6) (Fig. 1) (Hecht, 1998). Hydroxylation of NNK at the α-methyl carbon produces HPB and a DNA-pyridyloxobutylating intermediate (5), whereas the analogous reaction for NNAL generates diol, pyridyl-THF, and a pyridylhydroxybutylating species (8) (Fig. 1) (Hecht, 1998). NNK and NNAL are also oxidized at the pyridine nitrogen atom to form the respective N-oxides (Fig. 1) (Hecht, 1998).

Kinetic parameters for NNK bioactivation have been reported for a variety of P450s, including human P450s 1A1, 1A2, 2A6, 2A13, 2D6, 2E1, and 3A4; rabbit P450 2A10/2A11; rat P450 2B1; and mouse P450s 2A4 and 2A5 (Hecht, 1998; Felicia et al., 2000; Su et al., 2000; Zhang et al., 2002; Jalas et al., 2003). Importantly, the P450s with the greatest catalytic efficiency for NNK α-hydroxylation are members of the 2A subfamily and are expressed in the lung, for example, human P450 2A13 and mouse P450 2A5 (Felicia et al., 2000; Su et al., 2000; Zhang et al., 2002; Jalas et al., 2003). By contrast, human P450s 1A2, 2A6, and 3A4, which are predominantly expressed in the liver, are relatively poor catalysts of NNK bioactivation (Patten et al., 1996; Smith et al., 1996).

Given the ability of members of the P450 2A subfamily to efficiently catalyze the metabolic activation of NNK and the sensitivity of the rat lung to NNK-induced carcinogenesis, it was important to determine the kinetic parameters for an extrahepatic rat P450 2A3,

¹ Abbreviations used are: NNK, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone; diol, 1-(3-pyridyl)-1,4-butanediol; NNAL, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol; P450, cytochrome P450; OPB, 4-oxo-4-(3-pyridyl)butanal; lactol, 5-hydroxy-2-(3-pyridyl)tetrahydrofuran; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; pyridyl-THF, 2-(3-pyridyl)tetrahydrofuran; HPLC, high performance liquid chromatography; Spodoptera frugiperda.

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that is expressed in the lung (Kimura et al., 1989). Kinetic parameters for NNK metabolism were also determined using human P450 2A13 and compared with those for P450 2A3. Considering that NNK is rapidly converted to NNAL in vivo and that NNAL has similar carcinogenic potency to NNK in the rat lung (Hecht, 1998), an investigation of the kinetics of P450 2A3- and 2A13-mediated NNAL metabolism was warranted. Finally, the metabolism of individual NNAL enantiomers by P450s 2A3 and 2A13 was investigated because the metabolism and disposition of NNAL in animals and humans is profoundly affected by the absolute stereochemistry of the carbinol carbon (Hecht, 1998; Hecht et al., 2002; Wu et al., 2002). Thus, the effect of carbinol-carbon stereochemistry on the regioselectivity of metabolism by these P450s may provide important information about active-site architecture.

Materials and Methods

Caution: NNK and NNAL cause cancer in laboratory animals; they are to be handled with extreme care. Appropriate protective clothing and ventilation are to be used at all times.

Chemicals and Enzymes. NNK, (±)-NNAL, (R)-NNAL, (S)-NNAL, (±)-[5-3H]NNAL, (R)-[5-3H]NNAL, (S)-[5-3H]NNAL, and metabolite standards were synthesized as described (Jalas and Hecht, 2003). [5-3H]NNK (11 Ci mmol⁻¹, 98% radiochemical purity) was purchased from Moravek Biochemicals (Brea, CA) and purified to >99% radiochemical purity by reverse-phase HPLC. NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, sodium bisulfite, EDTA, and trichloroacetic acid were procured from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade commercially available. Microsomes were prepared from Spodoptera frugiperda (Sf9) insect cells that express cytochrome P450 2A3 or 2A13 (Liu et al., 1996). Human NADPH-cytochrome P450 oxidoreductase was purchased from PanVera (Madison, WI).

In Vitro Metabolism. Kinetic parameters for NNK metabolism were determined as described (Jalas et al., 2003), except that 5 mM sodium bisulfite was included in the incubation mixtures to trap OPB (no enzyme inhibition was observed under these conditions) (Peterson et al., 1991) and new HPLC conditions were developed (HPLC system I, see below). NNK was incubated with P450 2A3 or 2A13 (1 pmol) for 10 min at 37°C using substrate concentrations ranging from 0.25 to 50 μM (1 μCi) in a total volume of 200 μl. Kinetic parameters for NNAL metabolism were determined as described (Jalas and Hecht, 2003), except that HPLC system II was used (see below). NNAL was incubated with P450 2A3 (1.5 pmol) or 2A13 (3.5 pmol) for 20 (2A3) or 25 min (2A13) at 37°C using substrate concentrations ranging from 0.40 to 200 μM (1 μCi) in a total volume of 200 μl. Enzyme concentration and incubation time were varied in preliminary experiments to ensure that initial-rate conditions were employed in the kinetic determinations. Kinetic parameters were calculated by fitting the rate data to the Michaelis-Menten equation using the Enzyme Kinetics 1.1 module of SigmaPlot 7.0 (SPSS Science, Chicago, IL). Kinetic analyses were performed in quadruplicate (four data points per substrate concentration). The metabolism of (R)- and (S)-NNAL (1.0 μM, 1 μCi) was determined for P450s 2A3 and 2A13 exactly as in the kinetic studies.

HPLC Analysis. HPLC analysis of NNK metabolites (system I) was accomplished using a gradient from 92% A [20 mM sodium phosphate (pH 6.0) containing 1 mM NaHSO₃ and 1 mM EDTA] to 80% A over 30 min, and then to 50% A over 5 min; B was acetonitrile. HPLC analysis of NNAL metabolites (system II) was accomplished using a gradient from 94% A to 83% A over 70 min, and then to 50% A over 10 min; solvents were the same as described above, except that the mobile phase did not contain bisulfite. For both systems, the flow rate was 0.5 ml min⁻¹ and the scintillant flow rate was 1.5 ml min⁻¹ [Picofluor 40 (PerkinElmer Life Sciences, Boston, MA) for system I; Monoflow 5 (National Diagnostics, Atlanta, GA) for system II].

Results and Discussion

Steady-state kinetic parameters for rat P450 2A3- and human P450 2A13-mediated metabolism of NNK and NNAL were determined. Rat P450 2A3 catalyzed the α-hydroxylation of NNK to OPB and HPB at
maximal rates of 10.8 ± 0.4 and 8.2 ± 0.3 pmol min⁻¹ pmol P450⁻¹, respectively, with corresponding \( K_m \) values of 4.6 ± 0.5 and 4.9 ± 0.5 \( \mu M \) (Table 1). The ratio of \( \alpha \)-methylene to \( \alpha \)-methyl hydroxylation was 1.3:1 for P450 2A3, but was 3:1 for P450 2A13 (Table 1); this 3:1 ratio was similar to ratios reported previously (Su et al., 2000; Zhang et al., 2002). Human P450 2A13 was a slightly more efficient catalyst of \( \alpha \)-methylene hydroxylation than was rat P450 2A3, but had similar efficiency in catalyzing \( \alpha \)-methyl hydroxylation (\( V_{max}/K_m = 1.4 \pm 0.2 \) versus 1.7 ± 0.2 pmol min⁻¹ pmol P450⁻¹ \( \mu M \)⁻¹ for P450 2A3 and P450 2A13, respectively (Su et al., 2000; Zhang et al., 2002)). Only mouse P450 2A5 exhibits comparable \( V_{max}/K_m \) values; they range from 0.47 ± 0.09 (OPB) to 1.4 ± 0.1 (HPB) pmol min⁻¹ pmol P450⁻¹ \( \mu M \)⁻¹ (Jalas et al., 2003). By comparison, \( V_{max}/K_m \) values of 0.0085 and 0.0030 pmol min⁻¹ pmol P450⁻¹ \( \mu M \)⁻¹ were observed for human P450 2A6-mediated OPB and HPB formation, respectively (Patten et al., 1996). Other human hepatic P450s (e.g., P450 1A2, 2E1, and 3A4) metabolize NNK with catalytic efficiencies that are similar to or worse than those of human P450 2A6, the most efficient of NNK bioactivation currently known, and therefore, they may play a critical role in the generation of electrophylic DNA-damaging agents in vivo.

One caveat to bear in mind when comparing the catalytic efficiency of various P450s is that different expression systems and experimental conditions have been employed among various studies. In this regard, it is reassuring that the kinetic parameters determined in this study, in which P450 2A13 was expressed in S9 microsomes (Table 1), were quite similar to those determined previously using purified, reconstituted protein (Zhang et al., 2002). However, more work needs to be done in this area to validate comparisons among different expression systems.

### Table 1: Steady-state kinetic parameters for NNK and NNAL metabolism by cytochrome P450s 2A3 and 2A13

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolic Pathway</th>
<th>Substrate</th>
<th>( V_{max} ) (pmol product min⁻¹ pmol P450⁻¹)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max}/K_m )</th>
<th>( V_{max} ) (pmol product min⁻¹ pmol P450⁻¹)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat P450 2A3</td>
<td>( \alpha )-Methylene hydroxylation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NNK</td>
<td>10.8 ± 0.4</td>
<td>4.6 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>0.41 ± 0.01</td>
<td>3.8 ± 0.5</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-Methyl hydroxylation&lt;br/&gt;N-Oxidation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NNK</td>
<td>8.2 ± 0.3</td>
<td>4.9 ± 0.5</td>
<td>1.7 ± 0.2</td>
<td>0.98 ± 0.02</td>
<td>16 ± 1</td>
<td>0.061 ± 0.004</td>
</tr>
<tr>
<td>Human P450 2A13</td>
<td>( \alpha )-Methylene hydroxylation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NNK</td>
<td>13.8 ± 0.8</td>
<td>3.6 ± 0.7</td>
<td>3.9 ± 0.8</td>
<td>1.50 ± 0.05</td>
<td>36 ± 3</td>
<td>0.042 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-Methyl hydroxylation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NNK</td>
<td>4.6 ± 0.2</td>
<td>3.2 ± 0.5</td>
<td>1.4 ± 0.2</td>
<td>0.79 ± 0.02</td>
<td>40 ± 3</td>
<td>0.020 ± 0.002</td>
</tr>
</tbody>
</table>

N.D., not detected.

<sup>a</sup> When NNK was the substrate, the product was OPB. When NNAL was the substrate, the product was lactol.

<sup>b</sup> When NNK was the substrate, the product was HPB. When NNAL was the substrate, the product was diol.

In summary, steady-state kinetic parameters for P450s 2A3- and 2A13-mediated NNK and NNAL metabolism have been determined. NNK is clearly a much better substrate than NNAL for both P450s. To date, the \( V_{max}/K_m \) values for NNK bioactivation by rat P450 2A3 and human 2A13, along with mouse P450 2A5, are the highest among those that have been determined for expressed P450s. All three P450s are present in the lung of the respective species and thus may play critical roles in the induction of lung tumors in NNK-treated animals or humans exposed to tobacco products. Importantly, the similar triple and eightfold better catalyst than either 2A3 and 2A13, respectively, with corresponding \( K_m \) values of 1.7 ± 0.5 pmol P450⁻¹ \( \mu M \), respectively, with corresponding \( K_m \) values of 1.7 ± 0.3 \( \mu M \) (Jalas and Hecht, 2003). Thus, with regard to NNAL, \( \alpha \)-methylene hydroxylation, P450 2A5 is a 3-fold better catalyst than is 2A3 and an 8-fold better catalyst than is P450 2A13.

All three lung P450s, 2A3, 2A5, and 2A13, are much better catalysts of NNK metabolism than of NNAL metabolism. In light of these data, the tumorigenicity of NNK in the rat lung, which is similar to that of NNK (Hecht, 1998), may not be due to metabolic activation of NNK, but rather to the metabolic activation of NNK formed in vivo. Likewise, NNK may act as a carcinogen in humans not because it is metabolically activated, but rather because it has a long half-life (Hecht, 1998) and is converted to NNK, the preferred \( \alpha \)-hydroxylation substrate.

The metabolism of individual NNAL enantiomers by P450s 2A3 and 2A13 was investigated to ascertain the effect of carbonil-carbon stereochemistry on the regioselectivity of metabolism. Interestingly, (5)-NNAL was converted to NNK at a much greater rate than was (R)-NNAL by both P450s (Fig. 2). Also, (5)-NNAL, but not (R)-NNAL, was converted to its N-oxide by both P450s (Fig. 2). These results are consistent with those seen previously for mouse P450 2A5 (Jalas and Hecht, 2003) and may be a general feature of P450 2A-mediated NNAL metabolism across species. Notice that pyridyl-THF, a product of \( \alpha \)-methyl hydroxylation, was formed at greater rates when P450 2A3 was the catalyst than when P450 2A13 was used. The rate of pyridyl-THF formation is not entirely enzyme-dependent, however, as evidenced by a \( K_m \) value above 200 \( \mu M \) and rates of formation that do not increase linearly with respect to incubation time (data not shown) (Jalas and Hecht, 2003). The rate of formation of this metabolite may be determined by the stability of the diazonium ion precursor (8) and the competition between cyclization and hydrolysis to diol (Fig. 1). In any event, there clearly was not as dramatic an effect of substrate stereochemistry on rates of \( \alpha \)-hydroxylation as there were on rates of \( \alpha \)-oxidation and NNK formation. The effect of substrate stereochemistry on product distribution may provide insight into critical enzyme-substrate interactions leading to bioactivation/detoxification of NNAL and its oxidation to NNK.
kinetic efficiency of P450s 2A3 and 2A13 and the exquisite sensitivity for 20 (2A3) or 25 min (2A13) at 37°C and analyzed as described under Materials and Methods.

Substrates were incubated with P450 2A3 (1.5 pmol) or P450 2A13 (3.5 pmol) for 20 (2A3) or 25 min (2A13) at 37°C and analyzed as described under Materials and Methods.

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References


