Role of P450IIIE1 in the Metabolism of 3-Hydroxypyridine, a Constituent of Tobacco Smoke: Redox Cycling and DNA Strand Scission by the Metabolite 2,5-Dihydroxypridine

Sang Geon Kim and Raymond F. Novak
The Institute of Chemical Toxicology, Wayne State University, Detroit, Michigan 48201

ABSTRACT

The metabolism of 3-hydroxypyridine, a significant constituent of tobacco smoke, to 2,5-dihydroxypridine has been characterized in hepatic microsomes and in the reconstituted enzyme system using purified forms of P450. The redox cycling activity of the metabolite and its ability to damage DNA in vitro have been examined. Pyridine-induced microsomes, which contain elevated levels of P450IIE1 (Kim et al., J. Pharmacol. Exp. Ther., 246:1175-1182, 1988), catalyzed an 8-fold increase in the production of 2,5-dihydroxypridine, relative to control, which showed biphasic kinetics. Pyridine-induced rabbit hepatic microsomes exhibited a $V_{max}$ of 5.9 nmol 2,5-dihydroxypridine/min/mg protein and a $K_m$ value of 110 $\mu M$. In contrast, phenobarbital- and isosafrole-induced microsomes had $V_{max}$ values of 2.5 and 1.2 nmol/min/mg protein and $K_m$ values of 590 and 134 $\mu M$, respectively. Pyridine-induced rat hepatic microsomes also exhibited elevated catalytic activity toward the hydroxylation of 3-hydroxypyridine, with an 8-fold increase in $V_{max}$ (2.74 nmol/min/mg protein) relative to uninduced rat hepatic microsomes ($V_{max}$ = 0.34 nmol/min/mg protein). In the reconstituted system, cytochrome P450IIE1 displayed the greatest activity in the production of 2,5-dihydroxypridine of the major forms of rabbit P450 examined. P450IIE1 was 34-fold more active than P450IIB1 and 12-fold more active than P450IA2 in the production of 2,5-dihydroxypridine. The redox cycling activity of 2,5-dihydroxypridine has been characterized. The rate of NADPH oxidation in the presence of 0.5 $\mu M$ 2,5-dihydroxypridine was stimulated ~4-fold (69.2 nmol NADPH oxidized/min/mg protein), relative to control (16 nmol/min/mg protein). 2,5-Dihydroxypridine at 0.5 and 1.0 $\mu M$ produced a 12- and 17-fold increase, respectively, in the rate of superoxide anion production compared to control, as monitored by the SOD-inhibitable reduction of acetylated cytochrome c. 3-Hydroxypyridine alone failed to increase the rate of superoxide production. Inclusion of reduced glutathione in the incubation resulted in a pronounced decrease in the 2,5-dihydroxyprylene-stimulated rate of cofactor oxidation and superoxide production. The ability of 2,5-dihydroxyprylene to damage DNA was assessed by monitoring $\phi X$-174 DNA strand scission. The band intensity of the supercoiled form of DNA, when incubated with 1 $\mu M$ 2,5-dihydroxypridine, decreased substantially, with a concomitant increase in intensity of the band associated with the open circular form of DNA. The change in $\phi X$-174 DNA topology produced by 2,5-dihydroxyprylene was accelerated in a dose-dependent manner, with an estimated EC$_{50}$ of ~60 $\mu M$. Neither pyridine nor 3-hydroxyprylene produced DNA damage. These results show that P450 catalyzes the metabolism of 3-hydroxyprylene to 2,5-dihydroxyprylene, that P450IIE1 is the principal catalyst of 3-hydroxyprylene metabolism, and that the metabolite 2,5-dihydroxyprylene redox cycles and causes DNA strand scission.

INTRODUCTION

Numerous epidemiological and animal studies have established a strong correlation of alcohol and tobacco consumption with increased incidence of cancer (1-3). Indeed, the combination of smoking and drinking appears to be responsible for a majority of oral and pharyngeal cancer, as recently reported for a sizable population-based case-control study (3). One of many plausible mechanisms which underlie the increased risk of cancer associated with alcohol and tobacco consumption is the induction of microsomal enzymes (e.g., specific forms of cytochrome P450) that catalyze the metabolic activation of tobacco or other carcinogens.

Pyridine, 30 pyridine derivatives, 25 imidazoles, and 23 other nitrogen heterocyclic compounds have been identified among the 379 compounds present in cigarette smoke and smoke condensate, as revealed by extensive studies on smoke composition (4, 5). Ethanol, imidazole, various substituted imidazoles, and solvents such as acetone, trichloroethylene, benzene, and, more recently, pyridine have been shown to elevate the level of the alcohol-inducible form of cytochrome P450IIE1 (7-13) in hepatic and extrahepatic tissues, and P450IIE1 has been shown to be the low $K_m$ enzyme active in the conversion of pyridine to pyridine N-oxide (13).

It is well established that the cytochromes P450 are active in the metabolic conversion of many structurally diverse chemicals to electrophiles capable of reacting with cellular macromolecules. For example, the pyridine derivative methyprylon, which is a hepatocarcinogen in rats, was found to be metabolically activated by cytochrome P450 to a reactive species that binds DNA (14). Studies on alcohol-induced alterations in hepatic drug metabolism have revealed that chronic ethanol consumption leads to an increase in the content of hepatic microsomal cytochrome P450IIE1 (2, 15), which is involved in the bioactivation of various nitrosamines, including N-nitrosodimethylamine (16, 17) and N-nitrosopyrrolidine (18-20), to active carcinogens. The metabolism of the hepatocarcinogen N-nitrosopyrrolidine has been shown to be increased in microsomal fractions isolated from ethanol-consuming animals and to be catalyzed by isolated forms of cytochrome P450 (19, 20).

3-PYOH$^\dagger$ has been shown to be present in tobacco smoke and the content was estimated to be 130 $\mu g$/cigarette smoked (21, 22). Moreover, 3-PYOH and 2-PYOH have been reported to be teratogenic to 96-h chicken embryos (23). Although the metabolism of 3- and 2-PYOH has been characterized in microorganisms (24), little is known concerning the cytochrome P450-dependent metabolism or specificity of individual forms of P450 for these compounds. In the present paper, we report that 3-PYOH is converted to 2,5-DHPY by cytochrome P450 and that the alcohol-inducible form of cytochrome P450 exhibits a unique substrate specificity for this compound. Moreover, since 2,5-dihydroxyprylene can be converted to a quinone, the metabolism of 3-PYOH to 2,5-DHPY by cytochrome P450 is a plausible mechanism which may underlie the increased risk of cancer associated with alcohol and tobacco consumption.
none, which can redox cycle and generate reactive oxygen species, the formation of oxygen free radicals and induction of DNA damage by 2,5-DHPY have been characterized.

MATERIALS AND METHODS

Materials. 2-PYOH and 3-PYOH were obtained from Aldrich Chemical Company (Milwaukee, WI). 2,5-DHPY was kindly supplied by Dr. E. J. Behrman, Ohio State University (Columbus, OH). SOD, catalase, and common reagents were purchased from Sigma Chemical Company. OX-174RF DNA (form I) was purchased from BRL (Gaithersburg, MD). Rabbits were obtained from Lesser’s Rabbitry (Union Groves, WI).

Preparation of Microsomes. Male New Zealand white rabbits (2.0-2.5 kg) were given daily injections, for 5 days, of PY (100 mg/kg, i.p.) and fasted 18 h prior to sacrifice. No untoward toxicity was noted in treated animals. Control, PY-, PB-, and ISF-induced microsomes were prepared by differential centrifugation and stored at -70°C until used (7). Protein was assayed by the method of Lowry et al. (25) and cytochrome P450 content was determined according to the method of Omura and Sato (26). For rat microsomes, male Sprague-Dawley rats (200-250 g) were given daily injections, for 4 days, of PY (100 mg/kg, i.p.) and fasted 18 h prior to sacrifice. Rat hepatic microsomes were prepared as described above.

3-Hydroxyypyridine Hydroxylase Assay. 2,5-Dihydroxyypyridine formation was assayed by high performance liquid chromatography according to a modified method of Blauhoer and Paine (27), as described previously (12, 13). Incubation mixtures contained 1 mg microsomal protein, 3-PYOH (0.025-5 mM), 1 mM NADPH, and 0.1 mM potassium phosphate buffer (pH 7.5), in a total volume of 1.0 ml. Reactions were initiated by addition of NADPH after a 2-min preincubation period and allowed to proceed for 15 min at 37°C. Product formation was linear over the 15-min incubation period. The reactions were terminated by sequential addition of saturated Ba(OH)2, ZnSO4, and potassium phosphate buffer, pH 7.0, as described for the pyridine N-oxide assay. For high performance liquid chromatographic analysis of 2,5-DHPY, a mobile phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer (pH 7.4) was employed with a flow rate of 1.5 ml/min, using a Mobile Phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer (pH 7.4) was employed with a flow rate of 1.5 ml/min, using a guard column of CoPelhODS (Whatman; 10.0 x 0.5 cm).

Purification of Cytochromes P450. Cytochromes P450IIB1, IIE1, and IA2 were purified from rabbit hepatic microsomes according to previously published procedures (28, 29) and had specific contents of 17.5 units full scale). Concentrations of the metabolite were obtained from a mobile phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer, pH 7.0, as described for the pyridine N-oxide assay. For high performance liquid chromatographic analysis of 2,5-DHPY, a mobile phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer (pH 7.4) was employed with a flow rate of 1.5 ml/min, using a guard column of CoPelhODS (Whatman; 10.0 x 0.5 cm). Quantitation of 2,5-DHPY was accomplished at 320 nm using a LDS Spectrometer III variable wavelength detector (0.005 absorbance units full scale). Concentrations of the metabolite were obtained from a mobile phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer, pH 7.0, as described for the pyridine N-oxide assay. For high performance liquid chromatographic analysis of 2,5-DHPY, a mobile phase of 2.0% acetonitrile in 0.13 M potassium phosphate buffer (pH 7.4) was employed with a flow rate of 1.5 ml/min, using a guard column of CoPelhODS (Whatman; 10.0 x 0.5 cm).

RESULTS

Production of 2,5-DHPY in Rabbit Liver Microsomal Suspension. The saturation curves for the production of 2,5-DHPY from 3-PYOH in various rabbit hepatic microsomal suspensions are shown in Fig. 1. PY-induced microsomes, which contain an elevated level of P450IIE1, catalyzed a pronounced increase in the rate of hydroxylation of 3-PYOH, relative to uninduced microsomes, with saturation monitored at 0.5 mM substrate. PY-induced microsomes showed an approximate 9-fold increase in catalytic activity, compared to control microsomes, at a substrate concentration of 0.5 mM. Eadie-Scatchard analysis of the data revealed a Ka value of 110 ¿¿M and a Vmax value of 5.92 nmol 2,5-DHPY/min/mg protein in PY-induced

![Fig. 1. Saturation curves for the rate of 2,5-DHPY production from 3-hydroxyypyridine in uninduced (○) and PY- (●), PB- (△), and ISF-induced (▲) rabbit hepatic microsomes. Data points represent the mean ± SD of four determinations.](image-url)
rabbit hepatic microsomes (Table 1). Whereas ISF-induced microsomes also showed a high affinity monoxygenase \( K_m = 134 \ \mu M \) for the production of 2,5-DHPY, only a 1.6-fold increase in \( V_{\text{max}} \) relative to control microsomes was monitored. In contrast to PY- and ISF-induced microsomes, PB-induced microsomes exhibited a high \( K_m \) value of 590 \( \mu M \) and a \( V_{\text{max}} \) value of 2.49 nmol/min/mg protein, a 4-fold increase relative to control (Table 1). When metabolic rates were normalized for the different P450 levels present in the microsomal suspensions, an altered substrate specificity was clearly evident, with a \( V_{\text{max}} \) value of 3.02 nmol/min/nmol P450 for PY-induced microsomes, a value ~3- to 4-fold greater than that associated with control or PB-induced microsomal suspensions (Table 1). As shown in Fig. 1, 3-PYOH at 1 mM or higher resulted in a decreased rate of 2,5-DHPY production. This may have occurred as a result of either inactivation of the low \( K_m \) enzyme or substrate inhibition. When P450 levels were monitored after the incubation of PY-induced microsomes with 5 mM 3-PYOH at 37°C for 30 min, the total P450 content decreased by 24%, from 2.15 ± 0.04 nmol/mg protein to 1.63 ± 0.09 nmol/mg protein.

Production of 2,5-DHPY in Rat Liver Microsomal Suspensions. 3-PYOH hydroxylase activity was also examined in PY-induced rat hepatic microsomes over the substrate concentration range of 0.05 to 5 mM (Fig. 2). In PY-induced microsomes, the rate of 2,5-DHPY production increased with increasing 3-PYOH concentration, saturating at ~1 mM 3-PYOH (Fig. 2). Eadie-Scatchard analysis yielded a \( K_m \) value of 174 \( \mu M \) and a \( V_{\text{max}} \) of 2.74 nmol/min/mg protein. In contrast, uninduced rat hepatic microsomes exhibited relatively low catalytic activity toward the hydroxylation of 3-PYOH, with a \( K_m \) value of 139 \( \mu M \) and a \( V_{\text{max}} \) of 0.34 nmol/min/mg protein (Fig. 2). The \( V_{\text{max}} \) value for the production of 2,5-DHPY was elevated ~8-fold in PY-induced microsomes relative to control (Table 2). Although the absolute metabolic activities differed for the two species,

![Graph](image-url)

**Table 1** Kinetic parameters for 3-hydroxypyridine hydroxylation in rabbit hepatic microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{\text{max}} ) (nmol 2,5-DHPY/min/nmol P450)</th>
<th>( V_{\text{max}} ) (nmol 2,5-DHPY/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76 ± 12</td>
<td>0.58 ± 0.18</td>
<td>0.56 ± 0.18</td>
</tr>
<tr>
<td>665 ± 188</td>
<td>0.73 ± 0.08</td>
<td>0.72 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>PY-induced</td>
<td>110 ± 15</td>
<td>5.92 ± 0.39</td>
<td>3.02 ± 0.20</td>
</tr>
<tr>
<td>PB-induced</td>
<td>590 ± 112</td>
<td>2.49 ± 0.22</td>
<td>1.06 ± 0.09</td>
</tr>
<tr>
<td>ISF-induced</td>
<td>134 ± 11</td>
<td>1.20 ± 0.14</td>
<td>0.89 ± 0.10</td>
</tr>
</tbody>
</table>

![Graph](image-url)

**Fig. 2.** Saturation curves for the production of 2,5-DHPY from 3-PYOH in uninduced (C) and PY-induced (D) rat hepatic microsomes. Data represent the mean ± SD of four determinations.

![Graph](image-url)

**Table 2** Kinetic parameters for 3-hydroxypyridine hydroxylation in rat hepatic microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{\text{max}} ) (nmol 2,5-DHPY/min/mg protein)</th>
<th>( V_{\text{max}} ) (nmol 2,5-DHPY/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>139 ± 38</td>
<td>0.34 ± 0.02</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>PY-induced</td>
<td>174 ± 5</td>
<td>2.74 ± 0.06</td>
<td>1.41 ± 0.03</td>
</tr>
</tbody>
</table>

![Graph](image-url)

**Fig. 3.** Dixon plot of velocity (nmol 2,5-DHPY/min/mg protein) vs. P-Nitrophenol concentration showing competitive inhibition by PNP.

Inhibition of 2,5-DHPY Production by PNP. PNP has been shown to be metabolized primarily by P450IIE1 (35). Consequently, the effect of PNP on 2,5-DHPY production in PY-induced rabbit hepatic microsomes was examined at 3-PYOH concentrations of 0.05, 0.2, and 0.5 mM. Dixon plot analysis of the data (Fig. 3) revealed that PNP competitively inhibited the conversion of 3-PYOH to 2,5-DHPY with an extremely low \( K_i \) value of 6 \( \mu M \), thereby providing evidence for the role of microsomal P450IIE1 in the metabolism of 3-PYOH to 2,5-DHPY.

Reconstitution Studies. A reconstituted enzyme system consisting of P450, diaroylglycerol-3-phosphorycholine, and NADP-ctochrome P-450 reductase at pH 7.5 was employed to determine the turnover number for 2,5-DHPY formation. Purified rabbit P450IIE1 exhibited the largest turnover number of the isozymes examined; a turnover number of ~4.1 nmol 2,5-DHPY/min/nmol P450 was observed at a 5 mM 3-PYOH concentration. P450IIE1 was ~34-fold more active than P450IIIB1, which gave a turnover number of 0.12 nmol/min/nmol P450, and ~12-fold more active than P450IIB2, which produced 0.33 nmol 2,5-DHPY/min/nmol P450. P450IIB2 also functioned as a substrate and was metabolized to 2,5-DHPY by P450IIE1. The turnover number, however, was decreased by 60% from that monitored for 3-PYOH (i.e., ~1.6 nmol/min/nmol P450). P450IIIB1 did not show any detectable 2,5-DHPY production from 2-PYOH, and the turnover number of P450IIIB2 for the formation of 2,5-DHPY from 5 mM 2-PYOH was 0.15 nmol/min/nmol P450 (Table 3).

Stimulation of NADPH Oxidation by 2,5-DHPY. The stimulation of cofactor oxidation by 2,5-DHPY was examined. 2,5-DHPY (0.5 mM) stimulated the rate of NADPH oxidation over 4-fold, to 69.2 nmol/min/mg protein, while 3-PYOH failed to increase the basal rate of cofactor oxidation in PB-induced...
Table 3  Conversion of 3- and 2-hydroxypyridine to 2,5-dihydroxypyridine in the reconstituted enzyme system

Results are expressed as the mean ± SD of three determinations. The reaction mixtures contained 0.1 mM P450, 0.5 mM NADPH-cytochrome P-450 reductase, 30 μg/ml diilauroylglyceryl-3-phosphorylcholine, 0.1 M potassium phosphate buffer (pH 7.5), 1 mM NADPH, and 5 mM substrate. Reactions were initiated with NADPH at 37°C and incubated for 15 min with P450IIIE1 and for 30 min with P450IIB1 or P450IIA2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>P450 activity (nmol 2,5-DHPY/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PYOH (5 mM)</td>
<td>0.12±0.004</td>
</tr>
<tr>
<td>2-PYOH (5 mM)</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*The limit of detection in the assay.  
*ND, nondetectable.

Table 4  2,5-Dihydroxypyridine stimulation of NADPH oxidation in rabbit hepatic microsomes

Results are expressed as the mean ± SD of five to seven determinations.

<table>
<thead>
<tr>
<th>Addition</th>
<th>PB-induced microsomes (nmol NADPH oxidized/min/mg protein)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>16.0 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>2,5-DHPY (0.5 mM)</td>
<td>69.2 ± 3.1</td>
<td>433</td>
</tr>
<tr>
<td>+ GSH (0.1 mM)</td>
<td>26.0 ± 4.9</td>
<td>162</td>
</tr>
<tr>
<td>+ GSH (0.5 mM)</td>
<td>17.7 ± 1.6</td>
<td>110</td>
</tr>
<tr>
<td>3-PYOH (0.5 mM)</td>
<td>18.1 ± 1.4</td>
<td>113</td>
</tr>
</tbody>
</table>

Table 5  Stimulation of superoxide production in phenobarbital-induced rabbit hepatic microsomes by 2,5-dihydroxypyridine

Results are expressed as the mean ± SD of five to seven determinations.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Superoxide production (nmol/min/mg protein)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.0 ± 2.7</td>
<td>100</td>
</tr>
<tr>
<td>2,5-DHPY (0.5 mM)</td>
<td>97.8 ± 6.3</td>
<td>1222</td>
</tr>
<tr>
<td>+ GSH (0.1 mM)</td>
<td>25.7 ± 2.2</td>
<td>321</td>
</tr>
<tr>
<td>+ GSH (0.5 mM)</td>
<td>21.2 ± 2.3</td>
<td>265</td>
</tr>
<tr>
<td>2,5-DHPY (1.0 mM)</td>
<td>136.7 ± 5.2</td>
<td>1709</td>
</tr>
<tr>
<td>+ GSH (0.2 mM)</td>
<td>46.4 ± 7.6</td>
<td>530</td>
</tr>
<tr>
<td>+ GSH (1.0 mM)</td>
<td>20.4 ± 5.2</td>
<td>255</td>
</tr>
<tr>
<td>3-PYOH (1.0 mM)</td>
<td>9.2 ± 0.8</td>
<td>115</td>
</tr>
</tbody>
</table>

microsomes (Table 4). Inclusion of GSH (0.5 mM) in the incubation medium decreased the rate of cofactor oxidation stimulated by 2,5-DHPY to the basal level.

Superproduction by 2,5-DHPY. The SOD-inhibitable rate of reduction of acetylated cytochrome c in the microsomal incubation containing 2,5-DHPY was employed as an index of O2•− production. 2,5-DHPY at 0.5 and 1.0 mM increased O2•− production by 12- and 17-fold, respectively. 3-PYOH (1 mM) failed to affect the rate of O2•− generation (Table 5). GSH addition at 0.1 and 0.5 mM effectively decreased the rate of O2•− production by up to 90% (Table 5).

DNA Strand Scission by 2,5-DHPY. The effect of 2,5-DHPY on 8X-174 DNA topology was examined. 2,5-DHPY converted supercoiled 8X-174 DNA to the open circular form, as evidenced by a decrease in intensity of the supercoiled 8X-174 DNA band and an increase in intensity of the band associated with the open circular form (Fig. 4). Conversion of 8X-174 DNA to the open circular form by 2,5-DHPY occurred over the pH range 6.0 to 8.5. The change in 8X-174 DNA topology produced by 2,5-DHPY was concentration dependent (10 μM to 1 mM at pH 7.4), with an estimated EC50 of ~60 μM. Neither PY nor 3-PYOH at 1 mM caused DNA damage (Fig. 4). Catalase (0.5–1.0 μg) effectively inhibited the DNA strand scission produced by 1 mM 2,5-DHPY; in contrast, superoxide dismutase (1 μg) failed to protect DNA from damage (Fig. 5). Inclusion of GSH (1 mM) failed to protect against DNA damage produced by 2,5-DHPY. DNA damage also resulted from generation of 2,5-DHPY from 3-PYOH in a reconstituted enzyme system, which contained 1 mM NADPH. OC, SC, and LIN, open circular, supercoiled, and linear DNA, respectively.

Fig. 4. Concentration-dependent dX-174 strand scission by 2,5-DHPY. Lanes 1 through 6, 2,5-dihydroxypyridine at 0, 10, 50, 100, 500, and 1000 μM, respectively. Lanes A and B, 1 mM 3-hydroxypyridine and pyridine, respectively. OC, SC, and LIN, open circular, supercoiled, and linear DNA, respectively.

Fig. 5. Effects of catalase and SOD on 8X-174 DNA strand scission produced by 2,5-dihydroxypyridine. Catalase (0.5 μg) (lanes 1 and 2) in combination with 1 mM 2,5-DHPY protected against DNA damage; in contrast, SOD (1 μg) (lanes 3 and 4) in combination with 2,5-DHPY at 1 mM failed to exert a protective effect. OC, SC, and LIN, open circular, supercoiled, and linear DNA, respectively.

Fig. 6. dX-174 DNA damage resulting from metabolic conversion of 3-PYOH to 2,5-DHPY. Lanes 1 and 2, 0.1 mM P450IIE1, 0.5 mM NADPH cytochrome P-450 reductase, and 30 μg/ml diilauroylglyceryl-3-phosphorylcholine with 5 mM 3-PYOH. Lanes 3 and 4, 5 mM pyridine in the reconstituted enzyme system, which contained 1 mM NADPH. OC, SC, and LIN, open circular, supercoiled, and linear DNA, respectively.
DISCUSSION

Previous research in our laboratory has shown that PY is a potent inducer of P450IIE1 in both rabbit and rat hepatic microsomes and that this form of P450 functions as a low \\(K_m\) enzyme in the production of PY N-oxide from PY (12, 13). In order to determine whether such substrate specificity is uniquely restricted to PY or is applicable to other pyridine derivatives, studies on the form specificity for metabolism of 3-hydroxyppyridine were initiated. Given the numerous pyridine derivatives present in tobacco smoke and the relationship between alcohol consumption, smoking, and elevated rates of cancer, it was of interest to examine the metabolism of 3-PYOH, a significant constituent of tobacco smoke.

In the present communication, the formation of 2,5-DHPY from 3-PYOH is shown to be catalyzed by cytochromes P450, with P450IIE1 exhibiting the greatest metabolic activity of the major forms of P450 examined. This conclusion derives from results obtained using micromsomal suspensions, including competitive inhibition by PNP, and the reconstituted enzyme system. Although other forms of P450 catalyze the oxidation of 3-PYOH, substantially slower rates are seen and appreciably higher concentrations of substrate are required to produce concentrations of the metabolite, 2,5-DHPY, which can produce deleterious effects. Indeed, the dramatically elevated rate of 3-PYOH metabolism catalyzed by PY-induced microsomes at low substrate concentrations (as exemplified by data in Fig. 1) is striking in comparison to the other microsomal preparations and suggests that substantial metabolism of substrate, at the low levels normally encountered during smoking, will occur in tissues containing P450IIE1. Whereas the conversion of 2-PYOH to 2,5-DHPY also occurs, it is at a much slower rate than that monitored when 3-PYOH is the substrate. Although P450IIE1 exhibits activity towards 2-PYOH in the reconstituted system, 3-PYOH is the preferred substrate with the greater turnover number.

A distinct species difference in the metabolism of 3-PYOH to 2,5-DHPY also exists. Although the absolute rates of metabolism between the two species differ, with rabbit microsomes exhibiting 2-fold greater activity than rat liver microsomes, an approximate 8-fold difference in rates is monitored for PY-induced microsomes relative to control for both species.

It is interesting to note, however, that only PY-induced rabbit hepatic microsomes show decreased rates of metabolism at substrate concentrations above 0.5 mM. This appears to be the result of inactivation of the low \\(K_m\) enzyme primarily responsible for metabolism of 3-PYOH. This analysis is partially supported by the decreased P450 content monitored for the microsomal suspension following the incubation. Further mechanistic studies are required, however, to identify the basis of the inhibitory effect. Efforts to diminish the inhibition through decreased rates of metabolism (lower temperature) or use of elevated NADPH levels were largely unsuccessful. 2,5-DHPY, when added together with substrate, did not yield the characteristic inhibition (Fig. 1) observed for 3-PYOH metabolism, suggesting that redox cycling was not the primary mechanism for inhibition.

The metabolism of PY and conversion of 3-PYOH to 2,5-DHPY represent an interesting parallel to the benzene hydroxylation activity recently reported for P450IIE1 (8, 36). Acetone-induced rabbit hepatic microsomes exhibited a 5-fold increase in the hydroxylation of benzene to phenol and hydroquinone, and this increase correlated with the increase in IIE1 (36). Moreover, P450IIE1 was the only form of P450 to catalyze the production of significant quantities of hydroquinone (36). With phenol as a substrate, acetone-induced microsomes increased the rate of hydroquinone production 4- to 5-fold and, in the reconstituted system, P450IIE1 was 30-fold more active than the next most active form of P450 (36). One of the potentially toxic metabolites of benzene, benzenetriol, has been reported to generate superoxide and produce 0X-174 supercoiled DNA damage (34). The aforementioned results compare favorably with 2,5-DHPY production by P450IIE1 and, when viewed in toto, also suggest a P450IIE1 specificity for the metabolism of small aromatic ring systems (e.g., pyridine, benzene, phenol, para-nitrophenol, and aniline).

When the metabolite 2,5-DHPY was examined for its ability to damage DNA, a concentration-dependent alteration in \(\phi X-174\) DNA topology was monitored and an \(EC_{50}\) of 60 \(\mu M\) was estimated from the change in band intensity of the open circular form of \(\phi X-174\) DNA. In an effort to modulate DNA damage, catalase, SOD, and GSH were included with 2,5-DHPY and, of these, only catalase afforded protection, suggesting that \(H_2O_2\) may be required for DNA damage. The metal ion chelators deferoxamine and DTPA afforded some protection against DNA damage by 2,5-DHPY, whereas EDTA was without significant effect. These results suggest that, in the presence of 2,5-DHPY, metal ions and reactive oxygen species contribute to DNA damage, possibly as a result of 2,5-DHPY autoxidation. Further studies are required, however, to determine the mechanism(s) by which 2,5-DHPY causes DNA damage. To demonstrate that production of 2,5-DHPY from 3-PYOH by P450IIE1 could cause DNA strand scission, \(\phi X-174\) DNA was incubated in the presence of the reconstituted system, which contained either 3-PYOH or PY. A significant increase in DNA damage was noted for the system containing the substrate 3-PYOH. Thus, 2,5-DHPY, whether present singularly or generated from 3-PYOH by P450IIE1 in a reconstituted system, results in DNA strand scission in vitro.

The ability of P450IIE1 to catalyze the conversion of 3-PYOH to 2,5-DHPY had additional implications for toxicity and/or carcinogenicity. Since 2,5-DHPY can undergo conversion to a quinone, which may be mutagenic or carcinogenic (37), the redox cycling activity of this metabolite was examined. 2,5-DHPY stimulated a substantial increase in cofactor oxidation and \(O_2^-\) production. Since this redox cycling activity was catalyzed by microsomal NADPH-cytochrome P-450 reductase, it is possible that this process occurs via a one-electron reduction of the quinone to a semiquinone free radical. Alter-

![Fig. 7. Effects of iron chelators on \(\phi X-174\) DNA strand scission produced by 2,5-dihydroxyppyridine. Lanes 1 through 3, 1 mM 2,5-DHPY in the presence of iron chelators. Whereas EDTA (1 mM) (lane 1) had only a marginal effect, deferoxamine (1 mM) (lane 2) or DTPA (1 mM) (lane 3) resulted in the partial inhibition of DNA damage induced by 1 mM 2,5-DHPY. Lane 4, 1 mM 2,5-DHPY in the absence of iron chelators. Lane 5, \(\phi X-174\) DNA in the absence of 2,5-DHPY. OC and SC, open circular and supercoiled DNA, respectively.](image)
natively, the possibility of a two-electron reduction to the hydroquinone followed by autoxidation cannot be discounted. Reduced glutathione addition inhibited substantially (~90%) 2,5-DHPY stimulation of both cofactor oxidation and superoxide production. A plausible explanation for this inhibitory effect is the conjugation of the quinone with GSH, which served to decrease the availability of substrate for redox cycling and hence diminished the rate of cofactor oxidation and O$_2^-$ generation. Indeed, quinoid compounds have been shown to be capable of conjugating with nucleophiles such as GSH (38). The reaction between quinoid compounds and GSH has been reported to be a 1,4-reductive addition of the Michael type (39), and a prototypic nucleophilic addition reaction between GSH and 2-methyl-1,4-naphthoquinone has been described.

Since mono-, di-, and possibly trisubstituted GSH-quinone conjugates may be formed, additional experiments, beyond the scope of the present work, will be necessary to characterize these products. Nonetheless, these conjugates may be important in the toxicity and/or carcinogenicity of 2,5-DHPY. For example, GSH conjugates of bromobenzoquinone have been reported to be nephrotoxic (40) and subsequent research has revealed that 1,4-benzoquinone GSH conjugates are also toxic, with the greater toxicity associated with the more highly GSH-substituted products (41). The selective uptake of these conjugates into cells as the corresponding cysteine conjugate, mediated by γ-glutamyl transpeptidase, followed by their oxidation to the quinone has been implicated as the mechanism of toxicity of these conjugates. Since GSH conjugation of 2,5-DHPY occurs readily and is likely to result in GSH conjugates comparable in structure to those formed by bromobenzoquinone and benzoquinone, the potential for 2,5-DHPY-GSH conjugate(s) to produce toxicity/carcinogenicity in tissues may also exist and requires further evaluation.

In summary, evidence has been provided that the alcohol-inducible form of P450, P450IIE1, catalyzes the conversion of 3-PYOH, a significant constituent of cigarette smoke, to 2,5-DHPY. This metabolite redox cycles and stimulates the rates of cofactor oxidation and superoxide generation; DNA damage in vitro also results from production of this metabolite. A protein which is immunochromically reactive with antibody directed against P450IIE1 has been shown to be present in nasal and kidney tissue microsomes (42). Although chronic ethanol administration failed to induce this protein in nasal tissue, as monitored using immunochromic analysis, induction of IIE1 was demonstrated in kidney tissue (42). Moreover, nasal tissue has been shown to contain significant activity in the metabolism of a variety of xenobiotics (43-45) and preliminary results in our laboratory showed an increase in immunochromically detectable P450 protein using goat anti-rabbit IIE1 in nasal microsomes isolated from rats following inhalation exposure to pyridine. Elevation of P450IIE1 levels in hepatic or extrahepatic tissues through alcohol consumption, in conjunction with exposure to 3-PYOH and other nitrogen heterocycles such as pyridine that are present in tobacco smoke, may result in deleterious effects which contribute to an increased risk of cancer.

REFERENCES


5338
Role of P450IIE1 in the Metabolism of 3-Hydroxypyrididine, a Constituent of Tobacco Smoke: Redox Cycling and DNA Strand Scission by the Metabolite 2,5-Dihydroxypyrididine

Sang Geon Kim and Raymond F. Novak


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/17/5333

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.