Short Communication

Specificity of Cytochrome P450 2A3-Catalyzed α-Hydroxylation of N′-Nitrosonornicotine Enantiomers

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ABSTRACT:

N′-nitrosonornicotine (NNN) induces tumors in the rat nasal cavity and esophagus and is believed to be a causative agent for esophageal cancer in tobacco users. To exert its carcinogenic potential, NNN must be metabolically activated by α-hydroxylation at either the 2′- or 5′-carbon. We previously reported that the human cytochrome P450 (P450) P450, 2A6, efficiently and specifically catalyzed NNN 5′-hydroxylation. P450 2A3, which is expressed in the rat nasal cavity and to a small extent in the esophagus, is closely related to P450 2A6. P450 2A3, like 2A6, is a good catalyst of NNN α-hydroxylation (Km, 7 μM; Vmax 17 nmol/min/nmol). However, in contrast to P450 2A6, 2A3 catalyzed both 5′- and 2′-hydroxylation of NNN. The ratio of 2′- to 5′-hydroxylation was 1:3. These data, both with P450 2A6 and 2A3, were obtained using racemic NNN. P450 2A3 catalyzed metabolism of (S)-NNN occurred exclusively at the 5′-position. The predominant pathway of (R)-NNN metabolism was 2′-hydroxylation, and occurred to a 3-fold greater extent than did 5′-hydroxylation. These data are in contrast to those obtained from a recent study of (R)- and (S)-NNN metabolism by cultured rat esophagus. In that study, (S)-NNN was metabolized predominantly by 2′-hydroxylation and (R)-NNN equally by 2′- and 5′-hydroxylation. Taken together, these data provide strong evidence that P450 2A3 is not the rat esophageal P450 that catalyzes the metabolic activation of NNN. P450 2A3 may be an important catalyst of NNN activation in rat nasal mucosa.
Caution. NNN is a carcinogen and mutagen and therefore should be handled with extreme care, using appropriate protective clothing and ventilation at all times.

Results and Discussion

The products of P450 2A3-catalyzed metabolism of racemic [5-3H]NNN were analyzed by radioflow HPLC. Using HPLC system I (pH 7.0), only one radioactive metabolite peak was detected (data not shown). This peak coeluted with HPB and lactol, which do not separate in this system. When the reaction products were analyzed on a second HPLC system, two radioactive peaks that coeluted with lactol and HPB were detected (Fig. 2A). Lactol accounted for 75% of the metabolites, and the ratio of 2'-hydroxylation to 5'-hydroxylation was 1:3. When (S)-NNN was the substrate, the only metabolite detected was lactol, the product of 5'-hydroxylation. In contrast, HPB and lactol were both products of P450 2A3-catalyzed (R)-NNN metabolism (Fig. 2C). HPB was the predominant product. The ratio of 2'- to 5'-hydroxylation was 2.5:1.

Kinetic parameters for P450 2A3-catalyzed metabolism of racemic, (R)-, and (S)-NNN were determined and are presented in Table 1. The \( K_m \) for total \( \alpha \)-hydroxylation (2'- and 5'-hydroxylation) of racemic NNN was 13 \( \mu M \) and the \( V_{max} \) was 17.8 mmol/min/nmol of P450. Both (R)- and (S)-NNN were efficiently metabolized by P450 2A3 with \( K_m \) values between 5 and 19 \( \mu M \). P450 2A3 catalyzed the 5'-hydroxylation of (S)-NNN somewhat more efficiently than (R)-NNN, \( V_{max}/K_m \) values were 1.8 and 0.49, respectively. The \( V_{max}/K_m \) value for the 2'-hydroxylation of (R)-NNN was 1.1.

The relative affinity of P450 2A3 for (R)- and (S)-NNN is quite similar. However, the product distribution is strikingly different. An explanation for this is that (S)-NNN can only assume one orientation within the active site resulting in the oxidation of the 5-carbon, whereas (R)-NNN can assume two orientations allowing both 2'- and 5'-hydroxylation to occur. These data on the selectivity of oxidation of (R)- and (S)-NNN should be useful in modeling the active site of P450 2A3.

An earlier study (McIntee and Hecht, 2000) determined the relative rates of 2'- and 5'-hydroxylation of the two NNN enantiomers by cultured rat esophagus. In that study, which used 1 \( \mu M \) NNN, the (S)-enantiomer was metabolized by 2'-hydroxylation at a rate 6 to 8 times that of 5'-hydroxylation. (R)-NNN was metabolized equally
Kinetic parameters of P450 2A3-catalyzed metabolism of racemic, (R)-, and (S)-NNN

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>( K_m ) ( \mu \text{M} )</th>
<th>( V_{\text{max}} ) nmol/min/nmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Racemic NNN(^a)</td>
<td>Total ( \alpha )-hydroxylation</td>
<td>13.0 ± 1.7</td>
<td>17.8 ± 1.0</td>
</tr>
<tr>
<td>(S)-NNN(^b)</td>
<td>( 5' )-Hydroxylation</td>
<td>5.22 ± 0.61</td>
<td>9.34 ± 0.35</td>
</tr>
<tr>
<td>(R)-NNN(^c)</td>
<td>( 2' )-Hydroxylation</td>
<td>18.7 ± 3.8</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>(R)-NNN</td>
<td>( 5' )-Hydroxylation</td>
<td>11.3 ± 3.4</td>
<td>5.59 ± 0.43</td>
</tr>
</tbody>
</table>

\(^a\) NNN concentrations were 0.2, 0.4, 0.7, 0.8, 0.9, 1.7, 2.1, 3.2, 8, 15, 29, 34, 55, 74, 86, 103, 110, 190, and 360 \( \mu \text{M} \).

\(^b\) (S)-NNN concentrations were 0.3, 0.4, 0.6, 0.7, 1.1, 1.2, 1.3, 1.6, 1.9, 2.4, 9.7, 16, 48, and 94 \( \mu \text{M} \).

\(^c\) (R)-NNN concentrations were 0.2, 0.4, 0.7, 0.8, 1.2, 1.4, 1.6, 2.3, 3.5, 6.8, 8.8, 17, 43, 77, 91, 126, and 230 \( \mu \text{M} \).

**Fig. 2.** Radioflow HPLC analysis of the products of P450 2A3-catalyzed metabolism of racemic NNN (A), (S)-NNN (B), and (R)-NNN (C).

HPLC system II was used. P450 2A3 (2 \( \mu \text{mol} \)) was incubated with 1 \( \mu \text{M} \) [\(^5\text{-H}\)]NNN (3.4 Ci/mmol), or (S)- or (R)-[\(^5\text{-H}\)]NNN (3.0 Ci/mmol) for 10 min. The reaction with racemic NNN was analyzed directly on HPLC system II (panel A). The reactions with either (S)- or (R)-NNN were first analyzed on HPLC system I; the metabolite peak containing both HPB and lactol was collected and reanalyzed on HPLC system II (panels B and C).

**References**


Koeings LL, Peter RM, Thompson SJ, Rettie AE and Trager WF (1997) Mechanism-based metabolism in the esophagus. Previously, we reported that rat esophageal microsomes do not catalyze the 7-hydroxylation of coumarin, yet P450 2A3 efficiently catalyzes this reaction. Therefore, it appears that the level of P450 2A3 in the esophagus is insufficient to contribute significantly to NNN metabolism in this tissue. We have reached a similar conclusion for the metabolism of the potent esophageal carcinogen, \( \text{N}^\text{\alpha} \)-nitrosomethylbenzylamine (von Weymarn et al., 1999). Although P450 2A3 does not appear to be responsible for nitrosamine activation in the esophagus, it may contribute in the nasal cavity. The level of P450 2A3 mRNA in the rat nasal mucosa is more than 1000 times greater than that in the esophagus (Gopalakrishnan et al., 1999). P450 2A3 is a major P450 in rat nasal mucosa (Su et al., 1996). The metabolism of the NNN enantiomers has not been studied in the nasal mucosa; racemic NNN is metabolized by both \( 2' \)- and \( 5' \)-hydroxylation. Cultured rat nasal mucosa preferentially metabolized NNN by \( 2' \)-hydroxylation, and the ratio of \( 2' \)- to \( 5' \)-hydroxylation was between 2 and 3 (Brittebo et al., 1983). Microsomes from the nasal mucosa catalyzed \( 2' \)- and \( 5' \)-hydroxylation equally with a \( K_m \) of less than 3 \( \mu \text{M} \). P450 2A3 preferentially catalyzes \( 2' \)-hydroxylation of racemic NNN (Fig. 1A). Therefore, while P450 2A3 may contribute to the metabolism of NNN in the rat nasal mucosa, it appears that another P450 also plays a role.

In conclusion, the extrahepatic rat P450, 2A3, preferentially catalyzes the metabolism of racemic NNN by \( 5' \)-hydroxylation and exclusively catalyzes the \( 5' \)-hydroxylation of (S)-NNN. However, the hydroxylation of (R)-NNN by this P450 occurs preferentially at the \( 2' \)-carbon. This is in contrast to what was previously reported for the metabolism of (R)- and (S)-NNN by cultured rat esophagus (McIntee and Hecht, 2000), and thus is inconsistent with a significant role for P450 2A3 in the catalysis of NNN \( \alpha \)-hydroxylation in the rat esophagus.

**Table 1**

Kinetic parameters of P450 2A3-catalyzed metabolism of racemic, (R)-, and (S)-NNN.

**Fig. 2.** Kinetic parameters of P450 2A3-catalyzed metabolism of racemic, (R)-, and (S)-NNN.


