Metabolism of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in Mouse Lung Microsomes and Its Inhibition by Isothiocyanates

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ABSTRACT

The tobacco-specific carcinogen 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces lung tumors in rats, mice, and hamsters, and metabolic activation is required for the carcinogenicity. 2-Phenethyl isothiocyanate (PEITC), whose precursor gluconasturtiin (a glucosinolate) occurs in cruciferous vegetables, has been found to inhibit carcinogenesis by NNK. The purpose of the study was to investigate the enzymes involved in the metabolism of NNK in lung microsomes and to elucidate the mechanisms of inhibition of NNK metabolism by isothiocyanates. NNK metabolism in lung microsomes (isolated from female A/J mice) resulted in the formation of formaldehyde, 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol), 4-oxo-4-(3-pyridyl) butyric acid (keto acid), 4-(methyl nitrosamino)-1-(3-pyridyl)-N-oxide)-1-butanone, and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol, displaying apparent Kᵣ values of 5.6, 5.6, 9.2, 4.7, and 2540 μM, respectively. Higher Kᵣ values in the formation of formaldehyde and keto alcohol were also observed. When cytochrome P-450 inhibitors [2-(diethylamino)ethyl 2,2-diphenylpentanoate] hydrochloride (100 μM), carbon monoxide (90%), and 9-hydroxyelastone [hydrochloride, methimazole, and barium hydroxide were purchased from Sigma Chemical Company (St. Louis, MO). Zinc sulfate and Scintiverse LC

INTRODUCTION

NNK is a potent tobacco-specific carcinogen formed from the nitrosation of nicotine during tobacco processing and cigarette smoking (1-3). It has been found to induce tumors in the nasal cavity, lung, and liver of rats (4); the skin and lung of mice (5, 6); as well as the trachea, nasal cavity, and lung of hamsters (7). Metabolic activation of NNK is believed to involve the α-hydroxylation of either the methylene carbon leading to the formation of keto aldehyde and methyldiazohydroxide or the methyl carbon leading to the formation of formaldehyde and 4-(3-pyridyl)-4-oxobutyldiazohydroxide. The latter compound is a hypothetical precursor of the metabolite keto alcohol (Fig. 1) (8, 9). Generation of the diazohydroxides, which can cause DNA alkylation, may be responsible for the potent carcinogenicity of NNK. However, the enzymes that are responsible for the activation process are not well established. Devereux et al. (10) reported that a homologue of rabbit cytochrome P-4502 (cytochrome P-450IA1 or P-450c) and a 2,3,7,8-tetrachlorodibenzo-p-dioxin inducible cytochrome P-450 iso-enzyme (P450IA1 or P-450c) may be involved in the activation of NNK in Clara cells from rats.

Isothiocyanates are compounds that occur as glucosinolates in a variety of cruciferous vegetables such as cabbage and Brussels sprouts (11, 12). When the raw vegetables are wet and crushed, the glucosinolates are hydrolyzed by the plant enzyme myrosinase releasing glucose and forming isothiocyanates as products (11). BITC and PEITC are two naturally occurring isothiocyanates. PEITC at a daily dosage of 5 and 25 μmol/mouse for 4 consecutive d has been found to inhibit NNK-induced DNA adduct formation and tumorigenicity in mice (13). Administration of 1 mmol PEITC/kg body weight to rats significantly inhibited hepatic N-nitrosodimethylamine dealkylase activity (14). Recently, the inhibitory potency of a series of isothiocyanates on lung tumorigenesis has been found to be related to the alkyl chain length. 2-Phenethyl isothiocyanate, PPITC, and PBITC significantly inhibited lung tumorigenesis induced by NNK, whereas the shorter-chain isothiocyanates PITC and BITC were ineffective (15). The present study was undertaken to characterize the enzymes responsible for the metabolism of NNK in lung microsomes and to elucidate the mechanisms of inhibition by isothiocyanates.

MATERIALS AND METHODS

Chemicals. Unlabeled NNK, [5-3H]NNK (2.20 Ci/mmol; purity >98%), and [1H-methyl]NNK (1.06 Ci/mmol; purity >95%) were purchased from Chemsyn Scientific Laboratories (Lenexa, KS). PEITC, PITC, and BITC were purchased from Aldrich Chemical Company (Milwaukee, WI). PPITC was purchased from Fairfield Chemical Company (Blythewood, SC). PEITC and NNK metabolite standards were synthesized as described previously (8, 15). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP+, EDTA, magnesium chloride, methimazole, and barium hydroxide were purchased from Sigma Chemical Company (St. Louis, MO). Zinc sulfate and Scintiverse LC...
scintillation cocktail were obtained from Fisher Scientific Company (Fair Lawn, NJ). 9-Hydroxypyrido[2,3-d]pyrimidine was a gift from Pierre Lesca (Institut de Toxicologie, Toulouse, France). [2-(Diethylamino)ethyl]-2,2'-diphenylpentanone hydrochloride was a gift from Research Biochemicals (Wayland, MA). Monoclonal antibodies to rat cytochromes P-450IIB1 and -2, and P450IA1, polyclonal antibodies to rat cytochrome P-450IA2, and nonimmune serum were prepared and characterized as described previously (16–18).

Animals. Female A/J mice 5 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on an AIN-76A semipurified diet (ICN Biochemicals, Cleveland, OH) for 1 week. Mice were housed 10 per cage in polycarbonate cages on corn cob bedding and were maintained in an air-conditioned room with a 12-h light and dark cycle. For the experiment on the effect of PEITC in vivo, mice were given doses of 5 or 25 μmol/mouse in 100 μl of corn oil intragastrically 2 h prior to sacrifice.

NNK Metabolism Catalyzed by Lung Microsomes. Lung microsomes were prepared and isolated as described previously (13) and stored at −70°C. The cytochrome P-450 content and protein concentration were determined using previously described methods (19, 20). Unless otherwise stated, the incubation mixture consisted of 100 μM sodium phosphate, pH 7.4, 5 mM glucose-6-phosphate, 1.52 units of glucose-6-phosphate dehydrogenase, 1 mM NADP+, 1 mM EDTA, 3 mM MgCl2, 10 μM NNK (1 μCi of [5-3H]NNK and 1 μCi of [3H-methyl]NNK), and 0.1 mg of microsomal protein in 400 μl. The reaction mixture was incubated at 37°C for 30 min and terminated by the addition of 100 μl each of 25% zinc sulfate and saturated barium hydroxide. The sample was centrifuged and filtered through a 0.45 μm acetate filter disc (Fisher Scientific Company), and 200 μl were coinjected with 5 μl of NNK metabolite standards onto a reverse-phase HPLC system. The HPLC system consisted of a Waters automated gradient controller, two Waters 6000A pumps, Waters 710B WISP auto-injector, Waters 440 UV detector, and a C8 Bondapak column (3.9 x 300 mm; Waters, Milford, MA). It was eluted with a linear gradient of 95% A (0.02 M Tris-HCl buffer, pH 7.0) and 5% B (methanol) to 65% A and 35% B with authentic standards. Formaldehyde was identified by collecting the peak and subjecting the sample to HPLC analysis using mobile-phase buffers at pH 4, 4.5, 5, 6, and 7 as described by Cannella et al. (17). The identity of the metabolites was determined by coelution with authentic standards. Formaldehyde was identified by collecting the peak and coeluting it with a known amount of [3H]formaldehyde.

RESULTS

Identification of Metabolites. Metabolism of NNK in mouse lung microsomes led to the formation of formaldehyde, keto acid, NNK-N-oxide, keto alcohol, and NNAI (Fig. 2). The identities of the NNK metabolites were determined by coelution with authentic standards. Formaldehyde was identified by collecting the peak and coeluting it with a known amount of [3H]formaldehyde. Keto acid was further identified by collecting the peak and coeluting it with a known amount of [3H]formaldehyde. Keto alcohol was oxidized to keto acid or became bound to microsomal protein. Both metabolites were analyzed in the same assay. Since both metabolites were expected to be formed as a result of the oxidation of the α-methyl group (Fig. 1), the result was consistent with the theoretical ratio. A predicted NNK metabolite, keto aldehyde (8), was not observed under the assay conditions. This may be due to the possibility that keto aldehyde was oxidized to keto acid or became bound to microsomal protein.

Substrate Dependency of NNK Metabolism. In the substrate concentration range of 1 to 20 μM NNK, apparent Michaelis-Menten kinetics were observed in the formation of formaldehyde, keto alcohol, NNK-N-oxide, and keto acid (Fig. 3A). The apparent Kₘ and Vₐₙₐₓ values are summarized in Table 1. The formation of formaldehyde and keto alcohol had the same apparent Kₘ, 5.6 μM, and Vₐₙₐₓ, 57 pmol/min/mg protein, consistent with the idea that these two metabolites are formed from the same reaction. The formation of keto acid involved at least one more oxidation step, and an apparent Kₘ of 9.2 μM was observed. The Kₘ for the formation of NNK-N-oxide, 4.7 μM, was not significantly different from the Kₘ for formaldehyde and keto alcohol. However, the data do not allow us to conclude that the same enzyme was involved in the formation...
Table 1 Kinetic parameters for NNK metabolism in mouse lung microsomes

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>5.6 ± 0.9$^*$</td>
<td>57.2 ± 2.2$^*$</td>
</tr>
<tr>
<td>Keto alcohol</td>
<td>5.6 ± 0.9$^*$</td>
<td>56.0 ± 3.8$^*$</td>
</tr>
<tr>
<td>Keto acid</td>
<td>9.2 ± 1.0$^*$</td>
<td>4.2 ± 0.5$^*$</td>
</tr>
<tr>
<td>NNK-N-oxide</td>
<td>4.7 ± 0.9$^*$</td>
<td>54.2 ± 1.3$^*$</td>
</tr>
<tr>
<td>NNal</td>
<td>2540.7 ± 15.4$^*$</td>
<td>1322.3 ± 16.2$^*$</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD of four replications.

Table 2 Effects of enzyme inhibitors on NNK metabolism

<table>
<thead>
<tr>
<th>Inhibitor concentration (μM)</th>
<th>HCHO</th>
<th>Keto alcohol</th>
<th>Keto acid</th>
<th>NNK-N-oxide</th>
<th>NNal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF-525A$^*$</td>
<td>50</td>
<td>54 ± 5$^*$</td>
<td>58 ± 8$^*$</td>
<td>49 ± 2$^*$</td>
<td>63 ± 6$^*$</td>
</tr>
<tr>
<td>100</td>
<td>70 ± 2$^*$</td>
<td>72 ± 5$^*$</td>
<td>100$^2$</td>
<td>71 ± 2$^*$</td>
<td>29 ± 4$^*$</td>
</tr>
<tr>
<td>200</td>
<td>69 ± 6$^*$</td>
<td>71 ± 3$^*$</td>
<td>100$^2$</td>
<td>67 ± 7$^*$</td>
<td>26 ± 2$^*$</td>
</tr>
<tr>
<td>Carbon monoxide$^*$</td>
<td>50%</td>
<td>61 ± 4$^*$</td>
<td>62 ± 5$^*$</td>
<td>100$^2$</td>
<td>100$^2$</td>
</tr>
<tr>
<td>75%</td>
<td>76 ± 2$^*$</td>
<td>72 ± 2$^*$</td>
<td>100$^2$</td>
<td>100$^2$</td>
<td>31 ± 2$^*$</td>
</tr>
<tr>
<td>90%</td>
<td>100$^*$</td>
<td>100$^*$</td>
<td>100$^2$</td>
<td>100$^2$</td>
<td>32 ± 1$^*$</td>
</tr>
<tr>
<td>9-Hydroxyellipticine</td>
<td>1</td>
<td>30 ± 9$^*$</td>
<td>21 ± 6</td>
<td>30 ± 2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>34 ± 1$^*$</td>
<td>31 ± 2$^*$</td>
<td>47 ± 2$^*$</td>
<td>19 ± 1$^*$</td>
<td>13 ± 3$^*$</td>
</tr>
<tr>
<td>10</td>
<td>32 ± 6$^*$</td>
<td>30 ± 1$^*$</td>
<td>44 ± 2$^*$</td>
<td>22 ± 6</td>
<td>15 ± 1$^*$</td>
</tr>
<tr>
<td>Methimazole</td>
<td>100</td>
<td>15 ± 3$^*$</td>
<td>3 ± 2</td>
<td>100$^2$</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>500</td>
<td>20 ± 3$^*$</td>
<td>4 ± 3</td>
<td>100$^2$</td>
<td>23 ± 4$^*$</td>
<td>31 ± 7$^*$</td>
</tr>
<tr>
<td>1000</td>
<td>18 ± 0$^*$</td>
<td>0</td>
<td>100$^2$</td>
<td>20 ± 3$^*$</td>
<td>25 ± 4$^*$</td>
</tr>
</tbody>
</table>

* Microsomal incubation mixtures were preincubated with the inhibitors for 5 min. The formation of substrates was measured in the presence of NADPH. Values are the mean ± SD of three replications. Analysis of variance followed by the Newman-Keuls range test was performed on the original data; datum points with different superscripts in the same column are significantly different ($P < 0.05$) from the control and each other. For clarity only the number of percent inhibition is shown.

* SKF-525A, 9-[2-(diethylamino)ethyl] 2,2-diphenyl pentenoatehydrochloride.

Fig. 3. Substrate dependency of the metabolism of NNK. The incubation mixture consisted of microsomal protein (0.1 mg); a NADPH-generating system; 100 mM sodium phosphate buffer, pH 7.4; 1 mM EDTA; 3 mM MgCl₂; and 1–20 μM NNK (A) and 250–1000 μM NNK (B) (1 μCi of [5–H]NNK and 1 μCi of [3H-methyl]NNK) in a total volume of 400 μL. The reaction was initiated by the addition of substrate. Rates of metabolite formation were determined by analysis of variance followed by the Newman-Keuls range test. Points, mean of four replications; difference between the replication was less than 10%.
shown). To determine whether prostaglandin synthetase was involved in NNK metabolism, arachidonic acid (1 mM) was used in place of the NADPH-generating system. Metabolism of NNK was not observed (data not shown), suggesting that prostaglandin synthetase is not involved. The above results suggest that NNK is metabolized by cytochromes P-450, and that isozymes similar to cytochromes P-450IIIB1 and P-450IA1 appear to be involved.

**Inhibition of NNK Metabolism by PEITC.** A/J mice were given corn oil, 5 µmol of PEITC, or 25 µmol of PEITC intragastrically 2 h prior to sacrifice, and the activity of the lung microsomes in metabolizing NNK was analyzed. After a dose of 5 µmol of PEITC, the rate of the formation of formaldehyde, keto alcohol, and NNal decreased by approximately 30% and NNK-N-oxide by 40%. With a dose of 25 µmol of PEITC, formaldehyde, keto alcohol, and NNK-N-oxide, formation was decreased by 70–80% (Table 4). When the microsomes of the latter group of mice were used to study the formation of formaldehyde, keto alcohol, and NNK-N-oxide, in incubations containing 1–20 µM NNK, the Km was higher and the Vmax was lower than those obtained with the control microsomes (data not shown).

When 50 nm PEITC or PPITC was added to reaction mixtures of NNK metabolism assays, the isothiocyanates exhibited a mixed type of inhibition for keto alcohol formation (Fig. 4). A similar mechanism of inhibition by PEITC and PPITC was also observed for the formation of formaldehyde and NNK-N-oxide (data not shown). In the formation of both formaldehyde and keto alcohol, there was a competitive component of inhibition with apparent Ki of 90 and 30 nM (calculated from the slope of the double-reciprocal plot) for PEITC and PPITC, respectively. Since PEITC is known to react with amino, histidyl, and cysteinyl groups in protein, the noncompetitive component of the inhibition is probably due to this chemical reactivity.

In order to study the possible inhibition by metabolites of PEITC, the effect of preincubation of microsomes with PEITC in the presence and absence of a NADPH-generating system was studied (Table 5). The addition of 25 and 50 nM PEITC to the incubation produced the expected inhibitory effect. Preincubation of PEITC with microsomes resulted in even lower metabolic activity. However, the presence of the NADPH-generating system in the preincubation did not cause a further decrease in the metabolic activity. Similar results were found in an experiment in which control mouse lung microsomes were preincubated for 20 min with PEITC in the presence and absence of the NADPH-generating system and then diluted 20-fold for the NNK metabolism study (data not shown). The results suggest that PEITC directly binds to and inactivates the cytochrome(s) P-450 responsible for NNK metabolism, and metabolic activation of PEITC is not required for the inactivation.

**Relative Inhibitory Strengths of Different Isothiocyanates.** The effect of various isothiocyanates with different alkyl chain lengths on NNK metabolism is shown in Table 6. PITC at a concentration of 25 and 100 nM had the least effect on NNK metabolism. PBITC was the most effective in inhibiting NNK metabolism. With an increase in alkyl chain length, the inhibitory potency of the isothiocyanates increased.

**DISCUSSION**

The activation of NNK occurs by way of α-hydroxylation, which can result in the methylation and pyridyloxobutylation of DNA (1–3, 9). Microsomes are believed to be responsible for most of the oxidative metabolism of NNK in the cell. In the present work, products of α-hydroxylation, pyridine-N-oxidation, and carbonyl reduction were observed. The observed metabolite profile in lung microsomes was similar to that obtained with cultured A/J mouse lung (24). The presence of low and high Km forms of enzymes for the formation of formaldehyde and keto alcohol were observed. Both of these metabolites were
formed via the α-hydroxylation of the methyl carbon of NNK. The nature of the α-hydroxylation of the methylene carbon, which leads to the formation of a methylating agent, however, was not elucidated in the present work. By studying O-methylguanine formation in the rat lung following NNK doses of 0.1 to 100 mg/kg/d, Belinsky et al. (25) suggested that there were low and high Km pathways for the metabolism of NNK to a methylating agent. The present results provide further evidence for the presence of multiple Km pathways in the activation of NNK. Since the carcinogenic dosage of NNK is rather low, the low Km form of the enzyme should be more important in the activation of this carcinogen. Therefore, most of the present studies employed low NNK concentrations (10 μM).

Although results from studies with inhibitors should be interpreted with caution, the results in Table 2 suggest that cytochromes P-450 play a major role in the oxidative metabolism, but not in the reductive metabolism, of NNK. The results with methimazole suggest that the flavin-dependent monoxygenase is not important in the formation of keto alcohol but may be involved to a certain extent in the formation of keto acid and NNK-N-oxide. However, the possible nonspecific actions of the inhibitors made specific assignment difficult. The results with 9-hydroxyleptipicin also suggest that enzymes related to cytochromes P-450I1A1 and -2 are involved. However, because of the inter-species difference in the specificity and potency of the action of these inhibitors, additional studies are needed to substantiate this point.

The results obtained with antibodies (Table 3) suggested that, in mouse lung microsomes, enzymes immunochemically related to rat cytochromes P-450IIB1 and -2 can account for 25% of the activity for the formation of keto alcohol, whereas those to P-450I1A1 may account for 15% of the activity. Orthologues of P-450I1A2, if they exist in the mouse lung, did not play a role in NNK metabolism. However, the inter-species difference of immunoinhibition makes this interpretation less certain. The present results are different from those of Devereux et al. (10), who found that antibodies to rabbit cytochrome P-4502 (P-450I1B4, orthologous to rat P-450) inhibited DNA methylation by rat lung microsomes 83%, suggesting a key role of cytochrome P-450 in the activation of NNK. Several factors might contribute to the differences: (a) the relative importance of P-450 in NNK metabolism may be different between rats and mice; (b) Devereux et al. (10) studied the product of methylene oxidation, whereas we dealt with the products of the methyl oxidation; and (c) Devereux et al. (10) used 2 μM NNK, whereas we used 10 μM NNK as the substrate. It is possible that cytochrome P-450I1B1 is the major isozyme involved in the high Km pathway for NNK activation but not in the low Km pathway. The metabolism of NNK by purified rat liver P-450I1B1 in a reconstituted system displayed an apparent Km of 0.46 μM. However, the Km in a reconstituted system may be higher than the Km displayed by the same enzyme in microsomes.

The inhibitory action of PEITC is consistent with its inhibition of NNK-induced lung DNA methylation and carcinogenesis (13, 26). Two types of inhibition were demonstrated in the present work: (a) competitive inhibition due to competition between PEITC and NNK for the active site of the enzyme (most likely cytochrome P-450); and (b) a noncompetitive component of the inhibition possibly due to the chemical inactivation of cytochrome P-450 by PEITC. Both mechanisms should also be applicable in vivo; the relative importance depends on the time and dose of the exposure to PEITC and
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