ABSTRACT

Phenethyl isothiocyanate (PEITC), a naturally occurring chemopreventive agent, inhibits lung tumor induction in rats by the tobacco-specific nitrosamine 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK). In this study, we examined the mechanism of tumor inhibition by determining the effects of dietary PEITC on levels of NNK and its metabolites in various tissues of NNK-treated F344 rats. F344 rats were fed control or PEITC-containing diets (3 μmol/g diet) before and throughout NNK treatment. To study NNK metabolism and distribution under both short-term and chronic NNK+NNK+PEITC treatments, control and PEITC-treated groups were divided into four subgroups. Subgroups were treated with either a single injection of [5-3H]NNK (1.76 mg/kg) or a total of 12, 24, and 36 doses of NNK administered three times/week. After a final injection of [5-3H]NNK in each subgroup, the rats were sacrificed at various time intervals, and NNK and its metabolites in lung, liver, nasal mucosa, pancreas, kidney, stomach, and serum were measured. Time-course curves for the tissue metabolites were generated, and the areas under-the-curves were compared. We observed that lung, liver, and nasal mucosa target tissues of NNK carcinogenesis in F344 rats, were also the tissues that had the highest levels of α-hydroxylation metabolites relative to any of the other tissues. The metabolism of [5-3H]NNK in each tissue was increased by PEITC, and its carboxyl reduction metabolite, 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNAL). The most pronounced effect of PEITC was a reduction in levels of α-hydroxylation metabolites in most tissues examined (except nasal mucosa). The ratio of α-hydroxylation products to NNK+NNAL in most tissues was decreased by PEITC, indicating that α-hydroxylation of NNK/NNAL was inhibited. PEITC did not significantly affect the total levels of NNK and its metabolites in the lung and most tissues examined, indicating that PEITC does not alter the amount of NNK reaching the lung. These results support the hypothesis that inhibition of NNK-induced lung tumorigenesis by PEITC is a result of decreased metabolic activation of NNK.

INTRODUCTION

Cigarette smoking is an important cause of various cancers, particularly lung cancer (1). Nitrosamines, some of which are present in tobacco and tobacco smoke, are a class of compounds whose carcinogenic activities have been well established (2). NNK (Fig. 1) is one of the strongest carcinogenic nitrosamines present in tobacco and tobacco products (3, 4). NNK is a potent pulmonary carcinogen in laboratory animals, inducing lung tumors in rats, mice, and hamsters (3, 4). It also induces liver, nasal cavity, and pancreatic tumors in rats (3–6). The animal carcinogenicity data, as well as the detection of NNK-related hemoglobin and DNA adducts in smokers and NNK metabolites in the urine of smokers, support the role of NNK in tobacco-associated human lung cancer (5–10).

The carcinogenic effects of NNK are due to its metabolic activation by α-hydroxylation to electrophilic species that can react with DNA and other cellular molecules. The pathways of NNK metabolism have been extensively studied and are summarized in Fig. 1 (11–14). α-Hydroxylation leads to unstable intermediates that spontaneously decompose to either formaldehyde and 4-(3-pyridyl)-4-oxobutanediazohydroxide or to methanediazohydroxide and keto aldehyde. Methanediazohydroxide methylates DNA and 4-(3-pyridyl)-4-oxobutanediazohydroxide pyridyloxobutylates DNA (15, 16). The other pathways of NNK metabolism are: carbonyl reduction to give NNAL and oxidation of the pyridine ring, yielding NNK-N-oxide and 4-(methylamino)-1-(3-[6-hydroxy-3-pyridyl])-1-butanone. NNAL, the major metabolite of NNK, may also undergo α-hydroxylation, leading to DNA alkylation as shown in Fig. 1 and oxidation of the pyridine nitrogen, giving NNAL-N-oxide. NNAL can also be conjugated with glucuronic acid, leading to NNAL Gluc. Keto alcohol, keto acid, and hydroxy acid are secondary metabolites resulting from the α-hydroxylation pathways of metabolism.

A variety of dietary compounds are cancer chemopreventive agents (17, 18). Isothiocyanates are a class of these compounds that possess potent chemopreventive properties. PEITC, which occurs as a glucosinolate precursor in several vegetables (19) including watercress, inhibits NNK-induced pulmonary carcinogenesis in laboratory animals. When PEITC was fed in the diet (3 μmol/g) to F344 rats 1 week before and during the 20-week administration of NNK, it produced a 50% reduction in lung tumors (20). In A/J mice, administration of a single dose (5 μmol) of PEITC before NNK treatment resulted in a 62% reduction in lung tumors (21). Similar results were observed in mice treated with multiple doses of PEITC (22).

The mechanism of PEITC inhibition of NNK tumorigenesis has been studied. Initially, Morse et al. (20) found that PEITC inhibited the metabolic activation of NNK as measured by decreased methyl and pyridyloxobutyl DNA adducts in the lung. PEITC in vitro inhibits the metabolic activation of NNK in rat and mouse lung microsomes and in cultured rat lung and oral tissue (23–28). PEITC administered by gavage to rats and mice decreased metabolic activation of NNK by lung microsomes (23–26). Analysis at various time points following the PEITC dose revealed that the decrease in rat lung microsomes was persistent (25, 26). A different effect was observed in the liver. Liver microsomes from these rats showed an initial decrease, followed by an increase in metabolic activation of NNK 24 h after the PEITC dose. These data suggested that PEITC could induce hepatic metabolism of NNK. In related studies, the inhibition of NNK-induced lung carcinogenesis by indole-3-carbinol resulted from increased hepatic metabolism, with less carcinogen reaching the lung (29, 30). Therefore, we chose to examine the effects of PEITC on in vivo NNK metabolism to determine if PEITC inhibition of lung tumorigenesis was due to inhibition of metabolic activation of NNK in the lung and/or to induction of hepatic metabolism of NNK, thereby reducing the amount of NNK that reaches extrahepatic tissues. Thus, the current
study was designed to provide an overall picture of the effects of PEITC on in vivo NNK metabolism and tissue distribution, neither of which has been examined previously.

The protocol used for this study was based on the protocol used by Morse et al. (20) in the bioassay examining the effects of dietary PEITC on NNK-induced carcinogenesis in F344 rats. Therefore, we are examining PEITC effects under conditions known to result in inhibition of lung tumorigenesis. This study will also provide the first data on tissue distribution of NNK and metabolites in F344 rats under a chronic administration protocol used for tumor induction. In a previous study, this was examined only at one time point after a single dose of NNK (31).

MATERIALS AND METHODS

Chemicals. [5-3H]NNK (1.8 Ci/mmol) was purchased from Chemsyn Science Laboratories (Lenexa, KS). Purity was assessed by reversed phase HPLC and was 97%. Unlabeled NNK was supplied by Dr. Shantu Amin of the Organic Synthesis Facility, American Health Foundation. NNK metabolite standards were synthesized as described previously (32). [5-3H]NNK was diluted with unlabeled NNK to the desired specific activity. PEITC (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and its purity was confirmed by GC-MS.

Animals. Male F344 rats (150 g) were obtained from Charles River Breeding Laboratories (Kingston, NY). They were housed two to three per cage, were maintained under standard conditions (20 ± 2°C, 50 ± 10% relative humidity, 12 h light/dark cycle), and were given tap water ad libitum.

Animal Treatment. Treatment and dosing of the rats was based on the bioassay protocol that examined the effects of dietary PEITC on NNK-induced tumors (20). The protocol is summarized in Fig. 2. Eighty rats were divided into two groups of 40. One group (control) was fed an NIH-07 diet, and the other group (PEITC) was fed an NIH-07 diet containing 3 μmol PEITC/g of diet. The PEITC diet was prepared weekly and stored at 4°C before use. Under these storage conditions, PEITC was stable in the diet for at least 10 days (20). The rats were fed the diets ad libitum 1 week prior to carcinogen treatment and for the duration of the study.

The control and PEITC groups were divided into subgroups 1–4. Each subgroup was treated with NNK for different lengths of time. The NNK was administered by s.c. injection at a dose of 1.76 mg/kg body weight in 0.2 ml of saline. Body weights were determined weekly. Subgroup 1 (10 control rats and 10 PEITC treated rats) was administered a single s.c. injection of [5-3H]NNK (25 μCi; 1.76 mg/kg body weight). After the injection, the rats were sacrificed at different time intervals as described below. Subgroup 2 (10 control rats and 10 PEITC treated rats) was administered a single s.c. injection of [5-3H]NNK (25 μCi; 1.76 mg/kg body weight). After the injection, the rats were sacrificed at different time intervals as described below. Subgroup 2 (10 control rats and 10 PEITC treated rats) was administered unlabeled NNK (1.76 mg/kg body weight; s.c. injection in 0.2 ml saline) three times a week for 4 weeks. The final injection (12th dose) of the 4-week period was an injection of [5-3H]NNK at the same dose described above. The animals were sacrificed at various time intervals. Subgroup 3 (10 control rats and 10 PEITC treated rats) was administered unlabeled NNK (1.76 mg/kg body weight; s.c. injection in 0.2 ml saline) three times a week for 8 weeks. The final injection (24th dose) was [5-3H]NNK, and the animals were sacrificed. Subgroup 4 (10...
control rats and 10 PEITC-treated rats) received NNK for 12 weeks. The final injection (36th dose) was [5-3H]NNK, and the animals were sacrificed.

**Animal Sacrifice.** After the final injection of [5-3H]NNK, rats in subgroups 1 and 2 were sacrificed at 5, 10, 20, 30, and 60 min following the injection. Rats in subgroups 3 and 4 were sacrificed at 5, 15, 30, 60, and 120 min following the injection. Two rats were sacrificed/time point. At the appropriate time period, the blood was collected by cardiac puncture, and the rats were immediately decapitated. The lung, liver, pancreas, nasal mucosa, kidney, and stomach were quickly excised. The tissues were immediately frozen on dry ice and stored at −20°C until analysis. The blood was collected in a syringe containing EDTA and was transferred to EDTA containing Vacutainer tubes (Becton Dickinson, East Rutherford, NJ). The plasma was collected by centrifugation at 900 g for 10 min at 4°C and stored at −20°C until analysis.

**Determination of Animal Sacrifice Times.** A group of 21 male F344 rats (150 g) was treated with a single injection of [5-3H]NNK (1.76 mg/kg; 25 μCi) in 0.2 ml of saline. The rats were placed in metabolism cages for collection of urine. Urine was collected on dry ice. The rats were sacrificed at 0.5, 1, 2, 4, 8, 12, and 24 h following the injection, with three rats sacrificed/time point. No urine was collected from the 0.5-h animals, and urine from the 1- and 2-h animals was combined before analysis. The blood and tissues were collected as described above and analyzed as described below.

**Tissue and Serum Analysis.** The tissues were thawed in 1.15% KCl, blotted dry, and weighed. Tissues were minced with scissors and homogenized in 3–4 volumes of ice-cold 0.1 M HCl with an Ultra-Turrax T-25 homogenizer (IKA Works, Inc., Cincinnati, OH) at medium speed for 1–2 min. The homogenate was centrifuged at 10,000 × g for 15 min. The supernatant and pellet wash were concentrated. The residue was redissolved in 1 ml 20 mM sodium phosphate buffer (pH 7), sonicated for 10–15 mm, filtered through 13 mm 0.45 μm filters (PVDF LC13 filters; Whatman, Hillsboro, OR), and stored at −20°C until analysis by HPLC. The serum and urine samples were prepared for HPLC by filtration through Amicon Centrifree Micropartition filters (Amicon, Beverly MA). The amount of tissue sample injected/run was 200 μl for kidney, liver, and stomach, 500 μl for nasal mucosa samples, and 300–500 μl for lung and pancreas samples. The volume of serum analyzed/run was typically 200–300 μl. In the preliminary study to determine the appropriate animal sacrifice times, some tissues and serum from the later time points had to be combined and concentrated to reach detectable metabolite levels. Metabolite recoveries (calculated by spiking control tissue homogenates with known amounts of NNK and metabolites) varied between 65–78%.

NNK and metabolites in each sample were separated and quantitated by reverse phase HPLC on a Waters Associates system (Millipore; Waters Division, Milford, MA) equipped with either a model 440 UV detector or Perkin-Elmer 991 Photodiode Array set at a detection wavelength of 254 nm and a β-RAM radioflow detector (IN/US Systems, Inc., Fairfield, NJ). Separation was accomplished on a Rainin Microsorb C18 column with a 20 nm sodium phosphate buffer (pH 7)/methanol gradient increasing from 0–35% methanol in 70 min at 1 ml/min with a 15-min hold after the first 15 min. Metabolites were identified by coelution with known standards. Glucuronide conjugates...
were confirmed by collection of the peak of interest and treatment with β-glucuronidase (type VIII-A from Escherichia coli; Sigma Chemical Co., St. Louis, MO). Picofluor 40 (Packard Instruments, Meridan, CT) liquid scintillation cocktail was used in the radiolabel detector and was pumped at a flow rate of 3 ml/min.

**Data Analysis.** Metabolite levels in the tissues of each rat at each time point were measured. Two rats were sacrificed/time point, and an average was determined. The mean metabolite levels were plotted versus time, resulting in time course curves for each metabolite in each tissue. Five points (10 animals) defined a curve. The AUCs were determined using the trapezoidal rule. SEs for the AUCs were determined using the method of Baier (33), and AUCs obtained in the control groups were compared to AUCs obtained in the PEITC groups, according to the method of Baier (33). The difference between the control AUCs and the PEITC AUCs in each subgroup was determined to be significant if the observed critical value was greater than 2.57 (α = 0.05). The α-hydroxylation:NNK+NNAL ratio (Table 1) was calculated by adding the areas under the hydroxy acid, keto acid, and keto alcohol curves in a tissue and dividing that by the sum of the areas under the NNK and NNAL time course curves.

**RESULTS**

At the start of this study, it was not clear what sacrifice intervals should be used to obtain reasonable time course curves for NNK and metabolites in each tissue. There were no data available on the appearance and disappearance of NNK metabolites in rat tissues after either a single dose or chronic dosing. Ideally, we wanted to observe both a rise and fall in NNK and metabolite levels in each tissue. Therefore, a group of 21 rats was given a single injection of [5-3H]NNK, and the animals were sacrificed at 0.5, 1, 2, 4, 8, 12, and 24 h following the injection. Urine (when available) was also collected at these time points. NNK and metabolites in lung, liver, nasal mucosa, pancreas, kidney, stomach, and serum were quantitated as described in “Materials and Methods.”

The results of this experiment (data not shown) revealed that the metabolism of NNK was very rapid at this dose. Peak metabolite levels appeared to be reached at or before 30 min. After 30 min, metabolite levels dropped off rapidly, and most metabolites were near zero levels at ~2–4 h in most of the tissues examined. Urinary excretion of total radioactivity was also analyzed. A plateau (75–80% of total dose) was reached at about 4–8 h. This also indicates rapid metabolism of NNK. Based on these results, we chose to examine tissue metabolite levels at early time points following NNK administration in the NNK/NNK+PEITC study. Subgroups 1 and 2 (Fig. 2) were sacrificed at 5, 10, 20, 30, and 60 min following the single or final [5-3H]NNK injection. After collecting some of these data, we observed that a few metabolites were near peak levels at 60 min. Thus, we altered the times of sacrifice to 5, 15, 30, 60, and 120 min for subgroups 3 and 4. Because of these differences in sacrifice times as well as possible differences in recoveries among tissues and/or subgroups, interpretation of the data should focus on relative rather than absolute levels of metabolites in the various tissues and subgroups.

Unbound NNK and metabolites were extracted from lung, liver, nasal mucosa, stomach, pancreas, kidney, and serum of the rats in the different subgroups (Fig. 2). Data obtained from the livers of the rats in subgroup 4 are shown in Fig. 3 and are representative of the type of data obtained in other tissues and other subgroups. Rats were sacrificed at the different time points shown following [5-3H]NNK administration, resulting in time course curves for NNK and metabolites in each tissue. Each point on the graph is an average from two rats. Thus, there are five points or 10 rats defining each curve. A set of these graphs was obtained for each rat tissue in each subgroup. The areas under these curves were determined and are plotted in Figs. 4–6. The α-hydroxylation activity of a tissue was estimated by the ratio of α-hydroxylation products to substrate. This ratio was calculated by adding the areas under the hydroxy acid, keto acid, and keto alcohol curves in a tissue and dividing that by the sum of the areas under the NNK and NNAL time course curves. These ratios are shown in Table 1.

One of the first aspects examined in the this study was the tissue distribution of total unbound NNK-associated radioactivity (Fig. 4).

**Table 1** Ratios of α-hydroxylation metabolites to NNK + NNAL in tissues of rats treated with NNK or NNK and PEITC

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Subgroup 1 Control</th>
<th>Subgroup 1 PEITC</th>
<th>Subgroup 2 Control</th>
<th>Subgroup 2 PEITC</th>
<th>Subgroup 3 Control</th>
<th>Subgroup 3 PEITC</th>
<th>Subgroup 4 Control</th>
<th>Subgroup 4 PEITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>0.73 (*)</td>
<td>0.22</td>
<td>0.55</td>
<td>0.31</td>
<td>0.41</td>
<td>0.25</td>
<td>0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>Liver</td>
<td>1.2</td>
<td>0.41</td>
<td>1.3</td>
<td>0.80</td>
<td>1.8</td>
<td>0.92</td>
<td>0.28</td>
<td>1.3</td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>27</td>
<td>0.24</td>
<td>0.20</td>
<td>0.12</td>
<td>0.24</td>
<td>0.13</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.24</td>
<td>0.14</td>
<td>0.20</td>
<td>0.12</td>
<td>0.11</td>
<td>0.04</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>Serum</td>
<td>0.56</td>
<td>0.21</td>
<td>0.26</td>
<td>0.19</td>
<td>0.35</td>
<td>0.23</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.060</td>
<td>0.019</td>
<td>0.023</td>
<td>0.027</td>
<td>0.027</td>
<td>0.015</td>
<td>0.034</td>
<td>0.026</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.68</td>
<td>0.31</td>
<td>0.30</td>
<td>0.25</td>
<td>0.43</td>
<td>0.23</td>
<td>0.54</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* The ratios were calculated by dividing the sum of the areas under the hydroxy acid, keto acid, and keto alcohol time course curves by the sum of the areas under the NNK and NNAL time course curves.
EFFECTS OF PEITC ON TISSUE DISTRIBUTION OF NNK

Fig. 5. Areas (nmol g−1 min) under NNK and metabolite time course curves in lung, liver, nasal mucosa, and pancreas of control (□) and PEITC-treated rats (■). See Fig. 4 legend for details. *, the differences between the control AUCs and PEITC AUCs were significant. Significance was determined as described in "Materials and Methods."

Total nmol of NNK and metabolites were highest in the kidney, followed by stomach in all subgroups examined. Total levels of NNK and metabolites in the lung, liver, serum, pancreas, and nasal mucosa were comparable in all subgroups. PEITC treatment did not have any significant effect on the tissue distribution of NNK plus metabolites.

We then examined individual metabolites in each tissue. Areas under the lung metabolites time course curves are shown in Fig. 5. The secondary NNK metabolites resulting from the α-hydroxylation pathway (keto acid and hydroxy acid) were observed as were NNAL, NNAL-N-oxide, and NNAL Gluc. Some unmetabolized NNK was also present in the lung. NNAL was the predominant metabolite, comprising 41% of total metabolites in the control rats of subgroup 1 and increasing to 60% of the total in control subgroup 4 (after 12 weeks of chronic NNK administration). Keto acid and hydroxy acid comprised 33% of the total metabolites in the control rats of subgroup 1 (single dose of NNK). This decreased to 21% in subgroup 4 (12 weeks of chronic NNK dosing).

The PEITC-treated animals had lower levels of α-hydroxylation metabolites in the lung compared to controls in all of the subgroups, although the difference between the control and PEITC groups decreased with chronic NNK/NNK+PEITC administration. For example, the decrease in total α-hydroxylation products in the PEITC-treated animals compared to the control animals was 59% in subgroup 1 but only 38 and 31% in subgroups 2 and 3. In subgroup 4, there was only a 13% decrease of α-hydroxylation products in the PEITC-treated animals compared to controls, and the difference was determined to be not significant. NNAL-N-oxide levels were also decreased in the PEITC-treated animals, although the decrease was only significant in subgroups 1 and 2. The levels of NNAL were consistently slightly higher in the PEITC-treated animals, but the increases were not statistically significant. The ratio of α-hydroxylation products to NNK+NNAL is shown in Table 1. This ratio was lower in PEITC animals compared to controls in all of the subgroups. The largest difference between PEITC and control rats was observed in the subgroups administered a single injection of NNK (subgroup 1). Examination of the control rats only indicated that there was a noticeable decrease in this ratio as we moved from subgroups 1 to 4. The α-hydroxylation:NNK+NNAL ratio in the control rats in subgroup 1 was 0.73; this ratio decreased to 0.28 in the control rats of subgroup 4.
Fig. 6. Areas (nmol g⁻¹ min or nmol ml⁻¹ min) under NNK and metabolite time course curves in stomach, kidney, and serum of control (○) and PEITC-treated rats (■). See Fig. 4 legend for details. *: the differences between the control AUCs and PEITC AUCs were significant. Significance was determined as described in “Materials and Methods.”

The results obtained in the liver are shown in Fig. 5. The major metabolite in the liver was NNAL Gluc, which comprised 30% of the total metabolites in the control animals in subgroup 1. The NNAL Gluc percentages did not vary much between subgroups 1–4. Levels of α-hydroxylation metabolites and NNAL were also substantial. In subgroup 1 (control), hydroxy acid and keto acid were 22 and 16% of the total over the time period analyzed. After 12 weeks of chronic NNK treatment (subgroup 4), keto acid levels did not change, but hydroxy acid levels increased to 35% of the total in the control rats. NNAL percentages were 31% in control subgroup 1 and decreased to 17% in control subgroup 4.

PEITC-treated animals had significantly lower levels of α-hydroxylation metabolites relative to controls in all subgroups. In subgroup 1, hydroxy acid and keto acid levels were 54 and 56% lower, respectively, in the PEITC animals compared to controls. In subgroups 2–4, the decrease in α-hydroxylation metabolites in the PEITC rats was not as great when compared to the controls. Hydroxy acid levels were 43, 33, and 41% lower in the PEITC group compared to the control group in subgroups 2–4. Keto acid levels were decreased to a similar extent. There was a small but significant increase in NNAL Gluc levels in the PEITC rats compared to controls in all of the subgroups. This increase ranged from 18–33%, depending on the subgroup. There was a definite decrease in the α-hydroxylation:NNK+NNAL ratio in the PEITC animals as shown in Table 1. This ratio increased in both the control and PEITC-treated animals as we moved from subgroups 1–4.

Metabolite levels in the nasal mucosa are illustrated in Fig. 5. Hydroxy acid and keto acid comprised ~90% of the total metabolites. In the control rats of subgroup 1, 93% of the total metabolites were hydroxy acid and keto acid, with hydroxy acid predominating. In subgroups 2, 3, and 4, hydroxy acid + keto acid comprised 87, 89, and 88% of the total metabolites in the control rats. Hydroxy acid and keto acid levels were higher in the nasal mucosa (per g/tissue) compared to all other tissues examined except kidney. The α-hydroxylation: NNK+NNAL ratio (Table 1) in the nasal mucosa was 27 in the control animals of subgroup 1 (>25 times that seen in the lung and liver). PEITC had little effect on the levels of α-hydroxylation metabolites in the nasal mucosa.

AUCs for metabolites present in the pancreas are shown in Fig. 5. NNK, NNAL, and α-hydroxylation products were detected. NNK and NNAL were the predominant compounds in most of the subgroups. In
the control animals of subgroup 1, NNAL was 60% and NNK was 17% of the total metabolites. Keto acid comprised 13% of the total metabolites in the control animals of subgroup 1. The α-hydroxylation:NNK+NNAL ratio in the control rats decreased from 0.24 in subgroup 1 to 0.07 in subgroup 4. In the PEITC-treated animals, there were significant decreases in keto acid levels compared to controls in subgroups 1–3, respectively. Hydroxy acid levels were also lower in the PEITC animals in most subgroups, although the difference was only found to be significant in subgroup 2. The α-hydroxylation:NNK+NNAL ratios were lower in the PEITC groups compared to controls (Table 1).

NNK and NNAL comprised >90% of the total metabolites in the stomach as shown in Fig. 6. Metabolites resulting from α-hydroxylation were extremely low (<5%). When compared to other tissues, the stomach had the highest levels of NNAL. NNK levels were about two times higher in the stomach than in the kidney and were four to seven times higher than in all other tissues. Higher levels of NNK and NNAL were consistently observed in the PEITC-treated animals compared to controls, probably reflecting the overall decrease in metabolic activation of NNK observed in other tissues. The α-hydroxylation:NNK+NNAL ratio in the stomach was low (Table 1). In most cases, these ratios were lower in the PEITC animals.

High levels of NNK were present in the kidney, as can be seen by the high areas under NNK metabolite curves (Fig. 6). These levels were 6- to 10-fold higher than that observed in other tissues except stomach. Kidney NNK levels were 2–3 times higher than stomach NNK levels. The other major metabolites observed in the kidney were keto acid, hydroxy acid, and NNAL. Keto acid levels ranged from 16–27% of the total in the control animals of subgroups 1–4, whereas NNK levels accounted for 16–20% of the total metabolites. Hydroxy acid levels were 6–12% of the total in the control animals. The PEITC rats showed a trend similar to that seen in other tissues. There were significant decreases in the levels of α-hydroxylation metabolites in most of the subgroups of the PEITC animals when compared to the controls. Keto acid levels in the PEITC-treated animals were 46, 23, 40, and 50% lower than in controls in subgroups 1–4, respectively. NNK levels were in most cases higher in the PEITC-treated animals, particularly in subgroup 4, where NNK levels were 36% higher in the PEITC animals compared to controls, with the increase being significant.

The results of serum analyses are summarized in Fig. 6. NNK and NNAL were the major compounds detected in the serum. NNK levels ranged from 30–43% of the total (depending on the subgroup) in the control animals, whereas NNAL levels ranged from 28–36% of the total. Levels of α-hydroxylation metabolites in the control animals ranged from 18–28% of metabolites observed. PEITC-treated animals had significantly lower levels of α-hydroxylation metabolites compared to controls, but the difference between the groups decreased as we moved from subgroups 1–4.


discussion

In this study, we examined the type and amount of NNK and metabolites in various tissues of NNK-treated F344 rats and the effects of dietary PEITC on this tissue distribution. The effects of both short-term and chronic NNK/NNK+PEITC treatment on tissue metabolite levels were analyzed. The protocol was based on the protocol used by Morse et al. (20) in the NNK-PEITC bioassay in F344 rats. Therefore, we were analyzing the effects of PEITC under conditions that are known to result in lung tumor inhibition.

Because there were no data available on the change in tissue levels of NNK and metabolites with time in F344 rats, we performed a preliminary study to determine appropriate times for sacrificing the animals. Our preliminary investigations of NNK and metabolite tissue distribution (data not shown) indicated that metabolism was rapid. All metabolite levels in the tissues of interest reached peak levels between 20–60 min, and most metabolites declined rapidly after 30 min. Urinary analysis also indicated rapid metabolism, since ~80% of the dose was excreted within 4–8 h. NNK was found previously to have a short biological half-life in F344 rats, also indicating rapid metabolism (34). The change in tissue metabolite levels with time has never been examined in rats, but in hamsters administered a single dose of NNK intratracheally (35), plasma metabolite levels also reached a maximum in 15–30 min. Because of the rapid metabolism of NNK, we examined tissue metabolite levels at early time points following dosing.

A major finding in this study was that PEITC-treated animals had lower levels of α-hydroxylation metabolites in the lung (Fig. 5). This is consistent with the effects of PEITC on NNK pulmonary microsomal metabolism (23–26) and with the the inhibition of methyl and pyridyloxobutyl DNA adducts in the lung (20). The ratio of α-hydroxylation products to NNK+NNAL (Table 1) was used as an indication of the α-hydroxylation activity in each tissue. This ratio in the lung of PEITC-treated animals was lower than that in animals fed a control diet. These results indicate that PEITC inhibits metabolic activation of NNK in the lung and suggest that this inhibition of metabolic activation is the basis for the observed tumor inhibition.

In the liver, we also saw decreased levels of α-hydroxylation metabolites in the PEITC-treated rats (Fig. 5 and Table 1). NNAL Gluc levels were slightly increased in the liver of PEITC-treated rats, and NNAL levels were sometimes higher than in controls. There was no consistent change in the ratio of NNAL Gluc:NNAL, suggesting that the increased levels of NNAL Gluc are due to increased levels of NNAL and not to enhancement of hepatic UDP-glucuronosyltransferase activity.

One of our reasons for performing this study was to determine if PEITC could enhance hepatic metabolism of NNK, thereby leading to decreased lung bioavailability of NNK. Based on the analysis of total radioactivity in each tissue (Fig. 4), PEITC had little effect on the total levels of NNK and its metabolites in the tissues analyzed. These data, coupled with the decreased levels of α-hydroxylation metabolites and slightly increased levels of NNK/NNAL in the lung of PEITC-treated rats, suggest that PEITC does not affect the amount of NNK reaching the lung but does inhibit the metabolic activation of NNK in the lung. Analysis of the metabolites in the liver indicated that hepatic metabolism of NNK was not enhanced by PEITC. These data indicate that PEITC does not increase hepatic metabolism of NNK or alter lung bioavailability of NNK and support the proposal that PEITC inhibition of NNK-induced lung tumorigenesis is due to decreased metabolic activation of NNK in the lung.

In previous studies, Guo et al. (26) had observed an increase in oxidative microsomal metabolism in the liver of F344 rats 24 h following PEITC treatment and extensive induction in rat liver of cytochrome P450 2B1 by PEITC, which suggested that PEITC could enhance hepatic NNK metabolism. Cytochrome P450 2B1 is thought to be involved in NNK metabolism, although the extent of its involvement is not clear (36, 37). The results of the current study indicate a decrease in NNK oxidative metabolism by PEITC in rat liver. Since PEITC extensively induces cytochrome P450 2B1, it seems that this enzyme is not significantly involved in NNK metabolism in rat liver. Although Guo et al. (26) observed an increase in NNK oxidative metabolism at 24 h following PEITC treatment, a decrease was observed before 24 h. In the current study, the animals were fed PEITC in the diet, whereas in the study by Guo et al. (26), the animals were given a single dose of PEITC intragastrically. Because Guo et al. (26) observed decreased oxidative metabolism at early time points
following PEITC treatment, continuous exposure (as would be the case in the current study) may keep NNK metabolism rates at the decreased level. Also in the current study, the animals were treated with a combination of NNK and PEITC, whereas in the previous study, the rats were treated with PEITC only. It is possible that NNK can have some enzyme effects that modify the effects of PEITC. The effects of NNK on its own metabolism have never been examined (except in the current study, see below).

It is interesting that PEITC appeared to inhibit metabolic activation of NNK in the liver, yet Morse et al. (26) saw no significant effect of PEITC on NNK-induced liver tumorigenesis (20). PEITC was also found to have no effect on DNA methylation in rat liver, but there was a slight decrease in keto alcohol-releasing DNA adducts, which are indicative of pyridylloxobutylation of DNA. The role of these adducts in NNK-induced liver tumors is not clear. Analysis of metabolites in the present study cannot distinguish between the two pathways of NNK activation.

As stated above, PEITC-treated animals had lower levels of α-hydroxylation metabolites in the lung. Interestingly, after chronic administration of NNK/NNK+PEITC, the difference in α-hydroxylation metabolite levels in the lung between the control and PEITC-treated animals decreased (Fig. 5 and Table 1). After a single injection of NNK, there was a 59% reduction in α-hydroxylation metabolites in the PEITC group, but after 12 weeks of NNK/NNK+PEITC treatment, there was only a ~13% decrease. The α-hydroxylation:NNK+NNAL ratio decreased in control animals after chronic NNK treatment. It is not clear if this effect was due to chronic NNK treatment or to the difference in age of the animals (groups receiving 4, 8, and 12 weeks of NNK were also 4, 8, and 12 weeks older than the animals receiving a single NNK injection). It is also possible that multiple dosing of NNK led to progressively increased retention of unlabeled NNK, thereby affecting the ability of the animal to metabolize the tritiated compound. Because metabolic activation of NNK/NNAL was decreased in the control animals, PEITC effects were not as great. This decrease in metabolic activation in the control animals was observed in other extrahepatic tissues (pancreas and nasal mucosa) and serum. The opposite effect, however, was seen in the liver. There was an increase in NNK/NNAL metabolic activation with continued administration of NNK/NNK+PEITC, as judged by the increasing α-hydroxylation:NNK+NNAL ratio (Table 1).

In the nasal mucosa, another target tissue for NNK carcinogenicity, PEITC effects on α-hydroxylation metabolites were minimal. This result is consistent with the lack of an effect on NNK-induced nasal cavity tumors as well as DNA methylation and pyridylloxobutylation (20). In the nasal mucosa, we observed high levels of keto acid and hydroxy acid, products of α-hydroxylation. The α-hydroxylation:NNK+NNAL ratio (Table 1) was highest in the nasal mucosa compared to all other tissues examined. This value in the control animals receiving a single injection of NNK was 27 in the nasal mucosa, while the ratio was 1.2 in the liver and 0.73 in the lung. This indicates that the nasal mucosa has high NNK α-hydroxylation activity, which has also been observed in rat nasal microsomes and in cultured nasal mucosa (38–40).

We also investigated the type and amounts of metabolites present in the pancreas. NNK induces pancreatic tumors in rats (5, 6) when administered in drinking water, but recently rat pancreatic microsomes were found to lack the ability to α-hydroxylate NNK or NNAL (41). We did detect α-hydroxylation metabolites in the pancreas, but we do not know if they were formed in the pancreas or if they were formed elsewhere and transported to the pancreas. The α-hydroxylation:NNK+NNAL ratio was smaller in the pancreas than in other tissues known to activate NNK (Table 1). This suggests that if the pancreas has the ability to α-hydroxylate NNK, the activity is very low. Currently, there are no data available on the formation of NNK-induced DNA adducts in the pancreas. As was observed in other tissues, pancreatic levels of α-hydroxylation metabolites were lower in the PEITC groups. The effect of PEITC on NNK-induced pancreatic tumors is not known.

High concentrations of radioactivity were observed in the kidney and stomach. The high level in the kidney has been observed previously in NNK-treated F344 rats (31) and Syrian golden hamsters (35). It has also been seen in autoradiographic studies with some other nitrosamines and in other species (42, 43). The high concentration in the kidney may be a reflection of what is being excreted, since the major route of excretion is the urine (31). We found that NNK constituted the majority of radioactivity in the kidney, yet this study and others have only detected small amounts of NNK in the urine (12, 14, 31, 44). This probably indicates that the kidney reabsorbs NNK. The kidney is not known to activate NNK significantly. No methylated DNA adducts were detected in the kidney after a single dose of 40 mg/kg NNK or after 4 daily doses of 100 mg/kg NNK (45). Methylated DNA adducts in the kidney have only been detected after 12 daily doses of 100 mg/kg NNK. (46). Autoradiographic studies also indicate that NNK-associated radioactivity in the kidney is not due to bound metabolites (31). Therefore, most of the α-hydroxylation metabolites in the kidney were probably formed elsewhere and reached the kidney for excretion. As was observed in other tissues examined, PEITC-treated animals also had lower levels of α-hydroxylation metabolites in the kidney. This is probably due to the overall decrease in metabolism that was observed in the other tissues.

The high concentration of radioactivity in the stomach was composed primarily of NNK and NNAL. NNK and NNAL are basic compounds and most likely are trapped in the stomach after protonation. This was also observed in other studies (31, 47). Small amounts of α-hydroxylation metabolites were detected in the stomach. The α-hydroxylation:NNK+NNAL ratio was the smallest observed in the tissues examined. The stomach most likely has little capacity for metabolizing NNK/NNAL but rather acts as a temporary storage area for these compounds.

It is important to note that the highest α-hydroxylation:NNK+NNAL ratios in this study were observed in the nasal mucosa, liver, and lung, which are the target tissues in rats treated with NNK under the same dosing conditions. A high α-hydroxylation:NNK+NNAL ratio was also seen in the kidney, but a large portion of the metabolites observed in the kidney were probably formed in other tissues and reached the kidney for excretion. The major route of excretion of NNK-related metabolites is the urine (31). Also, as described above, previous studies indicate that the kidney does not activate NNK significantly (45, 46, 31). Therefore, the α-hydroxylation:NNK+NNAL ratio in the kidney is not an indication of what has specifically occurred in the kidney but reflects the metabolism of other tissues as well. The high α-hydroxylation:NNK+NNAL ratios in the nasal mucosa, liver, and lung are consistent with the levels of methylated DNA adducts in tissues of NNK-treated animals. Following a single dose of NNK, methylated DNA adducts were detected in the nasal mucosa, liver, and lung of NNK-treated rats but not in nontarget tissues such as heart, esophagus, spleen, and kidney (45). Autoradiographic studies also showed an accumulation of bound metabolites in the target tissues of NNK-treated animals, although some accumulation was also seen in nontarget tissues (47). The highest α-hydroxylation:NNK+NNAL ratios in the current study were detected in the nasal mucosa, followed by liver and then lung. This is also consistent with the levels of methylated DNA adducts, which were highest in nasal mucosa, followed by liver and lung (15).

In conclusion, we found that the major effect of PEITC was a reduction in the levels of α-hydroxylation metabolites of NNK in
most tissues examined. The α-hydroxylation:NNK + NNAL ratio in most tissues was lower in PEITC-treated animals, indicating that PEITC inhibited the α-hydroxylation of NNK. The tissue distribution of NNK plus metabolites was not greatly affected by PEITC, and in particular, PEITC did not alter the amount of NNK reaching the lung. These data indicate that PEITC inhibition of NNK-induced lung tumorigenesis is most likely due to decreased metabolic activation of NNK.

ACKNOWLEDGMENTS

We thank Terrence Baxter of the Research Animal Facility for assistance in performing the animal study. We also thank Neil Trushin for technical assistance in many aspects of this study.

REFERENCES

12. Morse, M. A., Eklind, K. I., Toussaint, M., Amin, S. G., and Chung, F-L. Characteristics of the α-hydroxylation of NNK plus NNAL in most tissues examined. The ca-hydroxylation:NNK+NNAL ratio in most tissues examined. The tissue distribution of NNK plus metabolites was not greatly affected by PEITC, and in particular, PEITC did not alter the amount of NNK reaching the lung. These data indicate that PEITC inhibition of NNK-induced lung tumorigenesis is most likely due to decreased metabolic activation of NNK. Carcinogenesis (Lond.), 13: 2205–2210, 1992.
Effects of Phenethyl Isothiocyanate on the Tissue Distribution of 4-(MethylNitrosamino)-1-(3-pyridyl)-1-butanone and Metabolites in F344 Rats

Marianne E. Staretz and Stephen S. Hecht


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/55/23/5580

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.