Evidence for an Important Role of DNA Pyridyloxobutylation in Rat Lung Carcinogenesis by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone: Effects of Dose and Phenethyl Isothiocyanate

Marianne E. Staretz, Peter G. Foiles, Lisa M. Miglietta, and Stephen S. Hecht

American Health Foundation, Valhalla, New York

ABSTRACT

The tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), selectively induces lung tumors in F344 rats. NNK is metabolically activated to intermediates that methylate and pyridyloxobutylate DNA. To explore the importance of pyridyloxobutyl DNA adducts in NNK-induced rat lung tumorigenesis, the first study in this report examined levels of these adducts in whole lung and pulmonary cells of F344 rats treated with different doses of NNK (0.3, 1.0, 10.0, and 50 mg/kg; 3 × weekly for 2 weeks). Pyridyloxobutyl DNA adducts were highest in Clara cells compared to alveolar Type II cells, alveolar macrophages, and small cells, suggesting that enzymes involved in the formation of the pyridyloxobutylating species are concentrated in Clara cells. When we compared lung tumor incidence at the different doses of NNK (S. A. Belinsky et al., Cancer Res., 50: 3772–3780, 1990) versus pyridyloxobutyl DNA adducts in Type II cells, we observed a significant correlation. Because NNK-induced lung tumors arise from the Type II cells, this suggests an important role for pyridyloxobutyl DNA adducts. In the second study presented in this report, we examined the effect of dietary phenethyl isothiocyanate (PEITC), an inhibitor of lung tumor induction in F344 rats by NNK, on O6-methyldeoxyguanosine (O6-mG) and pyridyl oxobutyl DNA adducts in whole lung and lung cells of F344 rats treated with NNK. F344 rats were fed control or PEITC-containing diets (3 μmol/kg diet) before and throughout NNK treatment (1.76 mg/kg, three times weekly for 4, 8, 12, 16, or 20 weeks). PEITC inhibited formation of pyridyloxobutyl DNA adducts in whole lung and all lung cells except macrophages. There was also inhibition of O6-mG, but it varied with cell type and length of NNK treatment. Overall, PEITC treatment decreased pyridyloxobutyl DNA adducts by 57% in Clara cells, 51% in Type II cells, 46% in small cells, and 44% in whole lung. PEITC treatment decreased O6-mG levels by 52% in Clara cells, 19% in Type II cells and small cells, and 36% in whole lung. These results support the hypothesis that PEITC inhibition of NNK-induced lung tumors is a result of decreased metabolic activation and DNA binding of NNK. The 50% reduction of pyridyloxobutyl DNA adducts in Type II cells agreed well with the 50% reduction of NNK-induced lung tumors by PEITC. Because NNK-induced tumors arise from Type II cells, these results suggest an important role for pyridyloxobutyl DNA adducts in NNK-induced rat lung tumorigenesis.

INTRODUCTION

NNK, a nicotine-derived nitrosamine, is a potent pulmonary carcinogen present in tobacco and tobacco smoke (1, 2). NNK induces lung tumors in mice, rats, and hamsters (1, 2). It is believed to play an important role in the induction of lung cancer in smokers (3). NNK is metabolically activated to intermediates that bind to DNA. The known pathways of NNK metabolic activation are shown in Fig. 1 (reviewed in Refs. 4 and 5).

α-Hydroxylation of NNK at the methylene carbon leads to 4-oxo-4-(3-pyridyl)-1-butanal and methanediazohydroxide, a DNA-methylating agent. One methyl adduct, O6-mG, has miscoding properties and is a critical factor in NNK induction of lung tumors in mice (6–9). It is also postulated to be important in NNK-induced lung tumors in rats (10). α-Hydroxylation of NNK at the methyl carbon leads to formaldehyde and 4-(3-pyridyl)-4-oxobutenediazohydroxide. This diazohydroxide reacts with DNA to form pyridyloxobutyl DNA adducts. Acid hydrolysis of pyridyloxobutylated DNA releases 50–80% of the adducts as HPB (11).

The lung is a complex, heterogeneous organ composed of over 40 cell types (12). The distribution of enzymes involved in the activation and detoxification of chemicals can vary among different lung cells (reviewed in Refs. 13 and 14). As a result, some lung cells may be more susceptible to the genotoxic effects of carcinogens. Early studies by Belinsky et al. (15) analyzed the distribution of O6-mG in the different lung cells of F344 rats treated with NNK. The highest concentration of O6-mG was found in the Clara cells, followed by alveolar macrophages, small cells, and alveolar Type II cells. These results suggested the presence of a high affinity enzyme in the Clara cells of rat lung capable of activating NNK to a methylating agent. Low levels of O6-mG-DNA methyltransferase were also detected in Clara cells, probably contributing to the accumulation and persistence of O6-mG (16). In a related study, it was observed that DNA methylation in the Clara cell was 50-fold greater by NNK than by N-nitrosodimethylamine, a weak lung carcinogen, indicating that the cell specificity of adduct formation contributed to the potent lung carcinogenicity of NNK (17). In a dose-response study, Belinsky et al. (15) found a significant correlation between levels of O6-mG in Clara cells of F344 rats and the incidence of NNK-induced lung tumors, suggesting a causative relationship (10). These results supported the role of O6-mG and its cell-specific accumulation in NNK-induced lung carcinogenesis in F344 rats. Interestingly although, Belinsky et al. (13) found that NNK-induced lung tumors arose from Type II cells, and no correlation was observed between Type II cell levels of O6-mG and lung tumor incidence. This apparent contradiction was not resolved.

All of these studies focused exclusively on NNK-induced methylation of DNA, but the relative importance of DNA methylation and pyridyloxobutylation in the induction of rat lung tumors was not clear. Several lines of evidence suggest that pyridyloxobutylation of DNA may also play an important role in NNK-induced lung carcinogenesis in F344 rats. Bioassays in F344 rats demonstrated that [methylene- D2]NNK and [methyl-D3]NNK were as carcinogenic toward lung as NNK (18). If DNA methylation alone were important, [methylene-D2]NNK should have been a weaker lung carcinogen than NNK. In addition, pyridyloxobutyl DNA adducts are highly mutagenic in bacterial and mammalian test systems (19, 20). Another interesting observation was that pyridyloxobutylated DNA was found to inhibit the repair of O6-mG, indicating that it can act as a cocarcinogen with a methylating agent (21).
In the first study presented in this report, we examined cell-selective DNA pyridylloxobutylation in F344 rats treated with various doses of NNK. The second study presented here focused on the effect of PEITC, an inhibitor of NNK-induced lung tumorigenesis (22), on lung cell levels of both O\(^6\)-mG and pyridylloxobutyl DNA adducts. When PEITC was fed in the diet 1 week before and during 20 weeks of NNK administration, it produced a 50% reduction in lung tumors (23).

The mechanism of PEITC inhibition of NNK lung tumorigenesis has been examined in several studies (reviewed in Ref. 22). The results indicated that PEITC decreases metabolic activation of NNK to pyridylloxobutylating and methylating species. Most of these studies have examined PEITC effects on in vitro and in vivo metabolites, which are products of the \(\alpha\)-hydroxylation pathways of NNK metabolism (24–28). Only one study examined the effect of PEITC on in vivo DNA adduct formation in rats; it inhibited formation of 7-methylguanine and pyridylloxobutyl DNA adducts (23). In that study, however, the promutagenic lesion \(O\(^6\)-mG was not measured. Only whole lung DNA was analyzed, which could give misleading results, because adduct levels in particular cells can vary. Also, only a short-term dosing protocol was used. The current study examined the effect of PEITC on \(O\(^6\)-mG and pyridylloxobutyl DNA adducts in whole lung as well as lung cell types (alveolar macrophages, alveolar Type II cells, Clara cells, and small cells). The protocol involved long-term administration (up to 20 weeks) of NNK and NNK + PEITC and was identical to that used in the NNK-PEITC bioassay in which PEITC inhibited NNK-induced lung tumors by 50% (23).

Animal Treatment in the Dose-Response Study. Groups of six rats were given s.c. injections of NNK dissolved in 0.9% saline at the following doses: 0 (saline control), 0.3, 1.0, 10.0, and 50 mg/kg body weight three times a week for 2 weeks. The rats were sacrificed 18 h following the final injection. Pulmonary cell separation was carried out as described below.

Animal Treatment in the PEITC Study. The protocol is summarized in Fig. 2. Rats were divided into two groups of 75. One group (control) was fed NIH-07 diet, and the other group (PEITC) was fed NIH-07 diet containing 3 \(\mu\)mol of PEITC per gram of diet (489 ppm). The PEITC-containing 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 (23). The rats were fed the diets \textit{ad libitum} 1 week prior to and throughout carcinogen treatment.

The control and PEITC groups were subdivided into five groups of 15, with each subgroup receiving NNK injections for different lengths of time. After 1 week on the appropriate diets, rats were given s.c. injections of NNK (in 0.9% saline) at a dose of 1.76 mg/kg body weight three times a week for 4, 8, 12, 16, or 20 weeks. Eighteen h after the final injection of NNK, the rats were sacrificed. Twelve rats per treatment group were used for pulmonary cell separation (two cell separation procedures, each with six rats) as described below. Whole lungs were removed from three rats per group.

Pulmonary Cell Separations. Lung cells were isolated as described (10, 15). Briefly, six rats per group were anesthetized with Nembutal (Abbott Laboratories, Chicago, IL). The lungs of the rats were perfused with HEPES (25 mM HEPES, 5 mM potassium phosphate, 5 mM dextrose, 5 mM KCl, and 150 mM NaCl, pH 7.4). The lungs were removed and lavaged to isolate macrophages. Small cells, Type II cells, and Clara cells were separated by centrifugal elutriation. The purity of cell fractions was determined as described (10, 15). The macrophage fraction was typically 98% macrophages. The small cell fraction (primarily endothelial cells and lymphocytes) was ~90% small cells. The purities of the Type II cell and Clara cell fractions were determined in each isolation procedure. The composition of the Type II cell fraction was typically 55–65% Type II cells, 1–10% Clara cells, and 25–44% small cells. The Clara cell fraction was typically 30% Clara cells, 10% Type II cells, 30% macrophages, and 30% small cells. DNA adduct levels in the different cell fractions were determined. Macrophage and small cell fractions were deposited into the Type II fraction. The purity of the Type II cell and Clara cell fractions were determined in each isolation procedure. The composition of the Type II cell fraction was typically 55–65% Type II cells, 1–10% Clara cells, and 25–44% small cells. The Clara cell fraction was typically 30% Clara cells, 10% Type II cells, 30% macrophages, and 30% small cells. DNA adduct levels in the different cell fractions were determined. Macrophage and small cell fractions were deposited into the Type II fraction. The purity of the Type II cell and Clara cell fractions were determined in each isolation procedure.
**DNA Isolation.** Lungs or lung cell pellets were homogenized in ice-cold 0.05 M Tris, 1 mM EDTA, pH 7.4 (10 ml per g whole lung tissue and 1–2 ml for the lung cell pellets). SDS was added to a concentration of 1%; proteinase K (Type XI) was added (200 μg/ml), and samples were incubated at 37°C for 60 min. Samples were extracted once with phenol (buffer saturated):Sevag (24:1 chloroform:isoamyl alcohol) and then once with Sevag. DNA was collected by ethanol precipitation and redissolved in 0.015 M sodium citrate, 0.15 M NaCl, 1 mM EDTA, pH 7.4 (2 ml for whole lung DNA and 1 ml for cell pellet DNA). Residual RNA was removed by treatment with RNase A (100 μg/ml) at 37°C for 30 min. Samples were then treated with proteinase K at 37°C for 30 min and extracted twice with Sevag. DNA was again collected by ethanol precipitation. The pellet was washed with ice-cold 70% ethanol and ethanol.

**Determination of Pyridyloxobutylated DNA (HPB-releasing Adducts).** Pyridyloxobutyl DNA adducts were measured by GC-MS, which quantitates the amount of HPB released upon acid hydrolysis (29). The only significant difference in the current assay was that the internal standard was added to the DNA samples before acid hydrolysis. For the assay, we used ~250–300 μg of whole lung DNA or 20–250 μg of lung cell DNA (depending on the particular cell type). After acid hydrolysis of the DNA, a 50-μl aliquot was removed for guanine determination as described previously (30).

**Determination of O6-mG.** O6-mG was measured by competitive ELISA using the O6A4 antibody (31). DNA was enzyme hydrolyzed by nuclease P1, phosphodiesterase I, and alkaline phosphatase (32). For the analyses, we used 500 μg of whole lung DNA or 100–500 μg of cell-type DNA. After hydrolysis, a 50-μl aliquot of each sample was removed for guanine quantitation. In the digested DNA samples, O6-mG was separated from normal deoxyribonucleosides by HPLC. The column used was a 250 × 4.6-mm Supelcosil LC-18S (Supelco, Bellefonte, PA). Solvent A was 10 mM potassium phosphate buffer (pH 5.6), and solvent B was 80:20 methanol:Solvent A. The flow rate was 1 ml/min, and the gradient was: 100% A for 3 min, 100–40% A in 35 min, 40% A-0% A in 10 min, held for 10 min at 100% B to wash the column, and then a return to initial conditions. A 3-min region surrounding the retention time of O6-mG was collected in silanized vials and dried under vacuum.

The procedure for the competitive ELISA was similar to that described previously (31). Briefly, ELISA plates were coated with O6-mG-BSA conjugate (10 ng/well). Plates were washed twice with PBS-Tween (0.05% Tween; Sigma) and three times with distilled water. HPLC fractions (or standards) were redissolved in 100 μl of 0.1% BSA in PBS. To these samples, 100 μl of the primary antibody (1:10,000 dilution) were added. Solutions were incubated at room temperature for 10 min, then 100 μl were added to triplicate wells of the ELISA plates. Plates were incubated for 1 h at room temperature and washed as above. Alkaline phosphatase-labeled goat antimouse antibody (KPL, Gaithersburg, MD) was added at a 1:3000 dilution (100 μl/well). Plates were again incubated 1 h at room temperature and washed as above. One hundred μl of a 1 mg/ml p-nitrophenyl phosphate solution in 1 M diethanolamine (pH 9.8) were added, and the absorbance at 405 nm was read after 45 min. The extent of inhibition of binding of the O6A4 antibody was converted to picomoles of O6-mG by a log-logit plot generated using synthetic O6-mG. All samples analyzed exhibited >20% inhibition. To determine the percentage of recovery of O6-mG in the samples, calf thymus DNA was spiked with known amounts of O6-mG and subjected to the same hydrolysis, HPLC, and ELISA procedures as above. The percentage of recovery range was 87–92%.

**RESULTS**

**Dose-Response Study.** We examined the levels of pyridyloxobutyl DNA adducts in whole lung and isolated lung cells of rats treated with doses of NNK ranging from 0.1 to 50 mg/kg. The results are summarized in Table 1. Levels of pyridyloxobutyl adducts were greatest in the Clara cells at all doses of NNK. They were approximately 3–10-fold higher than those observed in the other cell types. The formation of pyridyloxobutyl adducts was nonlinear with dose.

**Table 1. Dose-response for formation of pyridyloxobutyl DNA adducts in lung and isolated lung cells of rats treated with NNK**

<table>
<thead>
<tr>
<th>Dose of NNK (mg/kg)</th>
<th>pmol adduct/μmol guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole lung</td>
</tr>
<tr>
<td>0.3</td>
<td>1.2 ± 0.2*</td>
</tr>
<tr>
<td>1.0</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>10.0</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>50.0</td>
<td>12.1*</td>
</tr>
</tbody>
</table>

* Rats were treated with NNK at doses ranging from 0.3 to 50 mg/kg body weight three times weekly for 2 weeks. Eighteen h following the last injection, rats were sacrificed. Lungs from six rats were pooled, and lung cells were isolated. Whole lungs were removed from three rats per dose group (unless otherwise stated). Pyridyloxobutyl adducts were measured in DNA isolated from whole lung and isolated pulmonary cells as described in “Materials and Methods.”

* Mean ± SD from three rats.

* Average from two rats.
The efficiency increases in Type II cells, small cells, macrophages, 45-fold increase in pyridyloxobutylation efficiency was observed in the dose of NNK ranging from 0.3 to 50 mg/kg (three times weekly for 2 weeks). The efficiency of pyridyloxobutyl DNA adduct formation was calculated by dividing the level of pyridyloxobutyl adducts observed (pmol/μmol guanine) by the dose of NNK (mg/kg) and was plotted versus the dose of NNK.

The decrease in 6-mG levels was not observed consistently in the other cell types. In the PEITC-treated animals, Clara cell levels decreased 82% in the 12-week group. PEITC inhibited 6-mG levels in Clara, Type II, small cells, whole lung, and macrophages in the 8-week group, dietary PEITC inhibited 6-mG levels in Clara, Type II, small cells, whole lung, and macrophages by 62%, 40%, 60%, 58%, and 14%, respectively. However, different results were observed in the 16- and 20-week treatment groups. In these treatment groups, dietary PEITC resulted in ~70% inhibition of 6-mG in Clara cells but no inhibition in the Type II cells, small cells, and macrophages. In some cases, 6-mG levels were slightly higher in these cell types of the PEITC-treated rats in the 16- and 20-week treatment groups. There were 27% and 8% reductions in 6-mG levels in whole lung in the PEITC rats of the 16- and 20-week treatment groups. The AUCs shown in Fig. 5 (6-mG levels versus weeks of NNK treatment) were calculated using the trapezoidal rule. These AUCs are shown in Table 2. The percentage of inhibition by PEITC of 6-mG in Clara cells during the 20 weeks of NNK treatment was 53%, but the overall extents of inhibition in the Type II cells, small cells, and macrophages were only 18.8, 19.2, and 8.7%, respectively.

Levels of pyridyloxobutyl DNA adducts in the different lung cells and whole lung after 4–20 weeks of NNK treatment and the effect of dietary PEITC on these levels are shown in Fig. 6. As was observed in the dose-response study, the highest levels of pyridyloxobutyl adducts were observed in the Clara cells. This pattern did not change from 4 to 20 weeks of NNK treatment. Pyridyloxobutyl adducts in Clara cells increased somewhat from 4 to 20 weeks of NNK treatment, which was opposite to the effect seen with 6-mG. In the group of rats receiving NNK for 4 weeks, the level of pyridyloxobutyl adducts in the Clara cells was 25 pmol/μmol guanine (6- to 10-fold higher than that observed in whole lung and the other cell types analyzed). In the
Table 2 AUCs of the pmol O6-mG/µmol guanine versus weeks of NNK treatment
shown in Fig. 5

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>PEITC</th>
<th>% inhibition by PEITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clara cells</td>
<td>314</td>
<td>149</td>
<td>52.4</td>
</tr>
<tr>
<td>Type II cells</td>
<td>58.4</td>
<td>47.4</td>
<td>18.8</td>
</tr>
<tr>
<td>Small cells</td>
<td>18.7</td>
<td>15.1</td>
<td>19.2</td>
</tr>
<tr>
<td>Macrophages</td>
<td>184</td>
<td>167</td>
<td>8.72</td>
</tr>
<tr>
<td>Whole lung</td>
<td>27.4</td>
<td>17.4</td>
<td>36.5</td>
</tr>
</tbody>
</table>

* Calculated using the trapezoidal rule.

20-week treatment group, the level of pyridyloxobutyl adducts in the Clara cells was 40 pmol/µmol guanine (9–15-fold higher than that observed in the other cell types and whole lung). Pyridyloxobutyl adducts in macrophages, small cells, and Type II cells were similar, ranging from 2.1 to 5.0 pmol/µmol guanine. Adduct levels in these cell types and whole lung did not change greatly from 4 to 20 weeks of NNK treatment.

Dietary PEITC had noticeable effects on the cellular and whole lung levels of pyridyloxobutyl adducts. In the group of rats receiving NNK or NNK+PEITC for 4 weeks, there were 44, 56, 48, and 44% reductions of pyridyloxobutyl adducts by PEITC in Clara cells, Type II cells, small cells, and whole lung, respectively. The inhibition by PEITC was consistent through the 4–20 weeks of NNK treatment. In the 20-week treatment group, PEITC treatment resulted in a 70, 26, 58, and 48% inhibition of pyridyloxobutyl adducts in the Clara cells, Type II cells, small cells, and whole lung, respectively. In contrast, PEITC did not consistently inhibit pyridyloxobutyl adducts in macrophages. In most of the treatment groups, pyridyloxobutyl adducts in macrophages were slightly increased in the PEITC rats. Calculated AUCs for these data are shown in Table 3. The overall inhibition of pyridyloxobutyl adducts by PEITC in the Clara cells, Type II cells, small cells, and whole lung was 57, 51, 40, and 44%, respectively.

We can also compare the levels of the two types of adducts. As shown in Tables 2 and 3, pyridyloxobutyl DNA adducts were higher than O6-mG in most cell types and whole lung, when the whole period of NNK treatment is considered. Clara cell levels of pyridyloxobutyl adducts in control animals were ~1.7 times higher than O6-mG levels. Clara cell levels of O6-mG were higher than pyridyloxobutyl adducts after 4 weeks of NNK treatment but were lower thereafter. Pyridyloxobutyl adducts were also 2–3 times higher than O6-mG in small cells and whole lung. Type II levels of the two adducts were similar. Macrophages were the one cell type in which O6-mG levels were higher than pyridyloxobutyl adducts.

DISCUSSION

The first study described in this report examined cell-selective DNA pyridyloxobutylation in rats treated with various doses of NNK. Previous studies have only examined cell-selective NNK-induced DNA methylation. Because NNK can also pyridyloxobutylate DNA, we felt that a better understanding of NNK-induced lung carcinogenesis in rats also required the examination of pyridyloxobutyl adducts.

In this dose-response study, we observed that the levels of NNK-induced pyridyloxobutyl DNA adducts were highest in the Clara cells of rat lung at all doses of NNK analyzed. The Clara cell levels of
Thus, high levels of both methyl and pyridyloxobutyl adducts in the Clara cells could also be related to differences in repair of these adducts in Clara cells. This has also been observed in whole lung using a different dosing regimen (35). Previous studies also observed a nonlinear dose-response for \( O^\beta\)-mG using the same doses of NNK and dosing regimen, except that NNK was given for a total period of 4 rather than 2 weeks as in the current study (10). As suggested previously for formation of the methylating species, it appears that enzymes involved in the activation of NNK to a pyridyloxobutylating species are becoming saturated at the high doses of NNK (10). This dose-dependent difference in adduct formation was clear when the efficiency of pyridyloxobutylation was examined. Efficiency increased greatly from the highest to the lowest dose of NNK. The increase was greatest in the Clara cells (45-fold increase), but there was a marked increase (20–26-fold) in the other lung cells as well. In contrast, Belinsky et al. (10) found that the efficiency of methylation increased greatly (29-fold) in the Clara cells but only slightly in other cell types. It was suggested that a high affinity/low \( K_m \) pathway existed in the Clara cells for activation of NNK to a methylating species. Our data suggest that enzymes involved in formation of the pyridyloxobutylating species do not have as marked cell-specific differences in affinity for NNK. The different dose-response patterns in whole lung and lung cells we have observed with pyridyloxobutyl adducts compared to what Belinsky et al. (10) observed for \( O^\beta\)-mG are consistent with different forms of P450 enzymes being involved in methyl and methylene \( \alpha \)-hydroxylation of NNK (33).

Belinsky et al. (10) performed a dose-response study in which they determined the incidence of lung tumors in rats given doses of NNK ranging from 0.1 to 50 mg/kg three times weekly for 20 weeks. A significant correlation was observed (which was not observed in other cell types or whole lung), suggesting the importance of Clara cell levels of \( O^\beta\)-mG in NNK-induced rat lung tumorigenesis. However, ultrastructural examination of the tumors revealed that they arose from the Type II cells. Thus, there was an unresolved contradiction in the results. We measured pyridyloxobutyl adducts at the same doses of NNK for which lung tumor incidence was determined. Levels of pyridyloxobutyl adducts in the Type II cells were significantly correlated with lung tumor incidence (Fig. 4). This observation suggests that pyridyloxobutyl adducts play an important role in NNK-induced lung carcinogenesis in F344 rats. This is also supported by some of the results in the PEITC study.

The most noticeable effect in the PEITC study was the decrease in pyridyloxobutyl adducts in all cell types analyzed (except macrophages) and whole lung in the PEITC-fed rats. The decrease was consistent from 4 through 20 weeks of NNK treatment. The overall percentage of inhibition of pyridyloxobutyl DNA adducts by PEITC in Clara, Type II, small cells, and whole lung was 57, 50, 40, and 44% (Table 3). The inhibition of adduct formation in these cell types and whole lung is consistent with the 50% inhibition of NNK-induced lung tumors by PEITC (23) and again suggests an important role for pyridyloxobutyl adducts in NNK-induced rat lung carcinogenesis. It is

---

### Table 3 AUCs of the pmol pyridyloxobutyl adducts/μmol guanine versus weeks of NNK treatment shown in Fig. 6

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>PEITC</th>
<th>% inhibition by PEITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clara cells</td>
<td>548</td>
<td>235</td>
<td>57.0</td>
</tr>
<tr>
<td>Type II cells</td>
<td>65.6</td>
<td>32.4</td>
<td>50.6</td>
</tr>
<tr>
<td>Small cells</td>
<td>53.4</td>
<td>32.0</td>
<td>40.1</td>
</tr>
<tr>
<td>Macrophages</td>
<td>46.8</td>
<td>58.2</td>
<td></td>
</tr>
<tr>
<td>Whole lung</td>
<td>63.6</td>
<td>35.4</td>
<td>44.3</td>
</tr>
</tbody>
</table>

*Calculated using the trapezoidal rule.*
especially interesting that NNK-induced tumors are known to arise from Type II cells, and we saw a 50% inhibition of pyridyloxobutyl adducts in this cell type.

A slightly different PEITC effect was observed when O\(^6\)-mG levels were measured. PEITC treatment resulted in a 52% decrease of O\(^6\)-mG in the Clara cells (Table 2). However, in Type II cells and small cells, there was some inhibition by PEITC in the animals receiving NNK for 4, 8, and 12 weeks, but the inhibition disappeared in the 16- and 20-week groups. Thus, PEITC treatment resulted in an overall inhibition of O\(^6\)-mG of only 19% in Type II cells and small cells. This 19% inhibition of O\(^6\)-mG adducts in the Type II cells contrasts to the 50% inhibition of pyridyloxobutyl DNA adducts and does not agree well with the 50% reduction of NNK-induced tumors by PEITC. This suggests that pyridyloxobutyl adducts do play a predominant role in NNK-induced lung tumorigenesis in F344 rats.

Macrophages were the one type of lung cell where we did not observe PEITC inhibition of pyridyloxobutyl adducts and only a marginal inhibition of O\(^6\)-mG by PEITC. A pattern also seen with the macrophages is that they consistently had higher levels of O\(^6\)-mG compared to pyridyloxobutyl adducts. Isolated rat alveolar macrophages were shown to metabolize NNK, suggesting that the reactive species is generated in the macrophage (34). In that study, however, secondary \(\alpha\)-hydroxylation metabolites were measured, which cannot distinguish between methyl (leading to DNA pyridyloxobutylation) and methylene (leading to DNA methylation) \(\alpha\)-hydroxylation of NNK. Therefore, we do not know if methylene \(\alpha\)-hydroxylation rather than methyl \(\alpha\)-hydroxylation predominated in macrophages.

Macrophages from different species generally have lower levels of cytochrome P450 activity compared to Clara and Type II cells (13). But, previous studies and the current study found fairly high levels of O\(^6\)-mG in macrophages, suggesting that methanediolazozhydrxide or its \(\alpha\)-hydroxy-NNK precursor may be formed in other cell types and transported to the macrophage. PEITC is known to inhibit P450 enzymes involved in the activation of NNK (24, 26). The lack of inhibition of NNK DNA adducts in macrophages could indicate a non-P450-mediated activation of NNK in macrophages. A primary function of macrophages is the secretion of bioactive arachidonic acid derivatives such as prostaglandins and leukotrienes, which participate in inflammatory and immunoregulatory activities (36). Related to this function, macrophages are known to contain high levels of arachidonic acid compared to other lung cells and have lipoxgenase and cyclooxygenase enzyme activities (37, 38). Recently, lipoxygenase was found to be involved in NNK activation in human lung microsomes (39). It is possible that NNK activation in rat alveolar macrophages is primarily lipoxygenase mediated. Cytochrome P450s play a predominant role in rat lung activation of NNK, but because macrophages make up only ~4% of rat lung, non-P450-mediated metabolism due to macrophages could be masked when NNK metabolism by microsomes from whole lungs is examined. Greater numbers of macrophages are found in human lung (although the numbers are influenced by environmental factors, such as smoking) compared to rat lung, which may in part explain the detection of lipoxygenase-mediated NNK metabolism in human lung (39).

The effect we saw in macrophages illustrates a potential problem in using pulmonary alveolar macrophages in biomonitoring studies. It has been suggested that pulmonary alveolar macrophages, collected by bronchoalveolar lavage, can be used for monitoring chemoprevention clinical trials (40). Carcinogen-DNA adducts in pulmonary alveolar macrophages would be used to monitor the effectiveness of a chemopreventive agent. In our study, it is clear that the effects of PEITC were not accurately reflected in macrophage DNA adducts. Although PEITC inhibits NNK-induced lung tumorigenesis, there was no effect on adduct levels in the macrophages. The PEITC effect in macrophages was not an indication of what was occurring elsewhere (particularly target cells) in the lung. One must, therefore, exercise caution when choosing pulmonary alveolar macrophages for biomonitoring studies.

In this PEITC study, the animals were given NNK for different lengths of time (up to 20 weeks), allowing us to examine how long-term NNK administration may alter the levels and cellular distribution of NNK-induced adducts. We observed that pyridyloxobutyl adducts were not affected by long-term NNK administration. However, O\(^6\)-mG levels, particularly in the Clara cells, decreased markedly from 4 through 20 weeks of NNK administration. It is possible that age or chronic NNK treatment is affecting enzymes involved in the formation of the methylating species. In a previous study, we observed decreased metabolic activation of NNK in the lung after chronic (up to 12 weeks) NNK treatment, suggesting some effect of NNK on enzymes involved in NNK activation. The decrease in adduct levels we observed could also be related to some effects on the repair of the adduct. However, Belinsky et al. (10) found that NNK treatment inhibits the activity of O\(^6\)-mG-DNA methyl transferase in Clara cells (10). In that case, we would have expected O\(^6\)-mG levels in the Clara cells to increase with long-term NNK treatment. In addition, pyridyloxobutyl adducts have been shown to inhibit the repair of O\(^6\)-mG (21). Based on this observation, we would have again expected adduct levels to increase rather than decrease. The effect we observed was not consistent with what is known about repair of NNK-induced O\(^6\)-mG by O\(^6\)-mG-DNA methyltransferase.

In summary, pyridyloxobutyl DNA adducts as well as O\(^6\)-mG are present in the greatest amount in Clara cells, indicating that enzymes involved in both methyl and methylene hydroxylation of NNK are concentrated in the Clara cells. PEITC was found to consistently inhibit the formation of pyridyloxobutyl DNA adducts. There was also inhibition of formation of O\(^6\)-mG, but it varied with cell type and length of NNK treatment. These results support the hypothesis that PEITC inhibition of NNK-induced lung tumors is a result of decreased NNK metabolic activation and DNA binding in the lung. The results of these studies also suggest an important role for pyridyloxobutyl DNA adducts in NNK-induced rat lung carcinogenesis. This is based on the following: (a) NNK-induced tumors arise from the Type II cells (10); (b) in our dose-response study, we found a significant correlation between pyridyloxobutyl adducts in Type II cells and tumor incidence, which was not observed for O\(^6\)-mG (10); (c) in an NNK-PEITC bioassay, PEITC inhibited NNK-induced lung tumors by 50% (23), and using an identical protocol, we found that PEITC inhibited pyridyloxobutyl DNA adducts in Type II cells by 50%, whereas O\(^6\)-mG in Type II cells was only inhibited by 19%; (d) the 50% reduction of pyridyloxobutyl adducts in Type II cells agreed well with the 50% reduction in NNK-induced lung tumors (23). Collectively, these results suggest an important role for pyridyloxobutyl DNA adducts in NNK-induced rat lung carcinogenesis.

ACKNOWLEDGMENTS

We thank Stuart Coleman and Dominic Pullo for helping with the GC-MS analyses of HPB-releasing DNA adducts and Shelly Isaacs of the AHF Research Animal Facility for treatment of the animals. We also thank Steven A. Belinsky of the Inhalation Toxicology Research Institute, Albuquerque, NM, for initial instruction in the isolation of pulmonary cells.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 1997 American Association for Cancer Research.
Evidence for an Important Role of DNA Pyridyloxobutylation in Rat Lung Carcinogenesis by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone: Effects of Dose and Phenethyl Isothiocyanate

Marianne E. Staretz, Peter G. Foiles, Lisa M. Miglietta, et al.

Cancer Res 1997;57:259-266.