Human cervical tissue metabolizes the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, via α-hydroxylation and carbonyl reduction pathways

Introduction

Cervical cancer is the third most common malignancy in women worldwide and the second most common in developing countries (1). In the USA, cervical cancer is the third most common malignancy among Hispanics and the sixth most common cancer among white American women (2), there will be an estimated 12,800 new cases and 4600 deaths from this disease in 2000 (3). Cervical cancers are generally thought to develop from dysplastic precursor lesions termed cervical intraepithelial neoplasias (CIN). These lesions are graded as 1–3 and carcinoma in situ (CIS), based on the degree of dysplasia.

Epidemiological studies have demonstrated that several factors, including infections with high-risk human papillomaviruses (HPVs), such as HPV 16 and HPV 18, constitute a very important risk factor for invasive cervical cancer and for its precursor lesions (4,5). However, given the high frequency of infections in the general population, estimated to be ~50% in young sexually active women (6), it has been suggested that viral infection by itself is not a sufficient cause for neoplastic conversion. In addition, the long incubation period between initial infection and development of cancer, as well as the frequent regressions of most HPV-induced dysplasias, indicate that besides HPV infection, other genetic damage is required for the development of this malignancy (7–10).

Naguib et al., in 1966 (11), found a correlation between smoking and cervical cancer. In 1990, Warren Winkelstein reviewed 18 studies on cigarette smoking and cervical cancer (12). After adjusting for all other known risk factors, 15 of these studies supported the conclusion that women who smoke cigarettes displayed a 4.3-fold higher risk of developing this cancer (12). Recent studies further support the association between cigarette smoking and cervical disease (13–15). In a cross-sectional study of 77 Brazilian women with biopsy-confirmed CIN, smokers were found to be at 6.6-fold greater risk of developing CIN 2 and 3 than nonsmokers (13). Kjaer et al. found a dose–response relationship between smoking and cervical CIS in Danish women (16). Exposure to environmental tobacco smoke has also been shown to increase the risk of cervical cancer (17).

Recently, we demonstrated that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P), carcinogens found in tobacco smoke, were present in significantly higher concentrations in the cervical mucus of smokers than in nonsmokers (18,19). Moreover, significantly higher concentrations of B[a]P-induced DNA adducts were detected in the cervical tissue of smokers than in nonsmokers (19). Whether NNK can induce DNA adducts in the cervical tissue of smokers remains to be determined.

NNK is a potent carcinogen in laboratory animals. It requires metabolic activation to exert its carcinogenic activity (reviewed in ref. 20). Of major importance in NNK metabolic activation is the α-hydroxylation pathway, catalyzed by various cytochrome P450 enzymes. This pathway leads to the formation of reactive...
intermediates capable of reacting with cellular macromolecules, including DNA. In rodents, α-hydroxylation of NNK contributes to ≥50% of its total metabolites (20). Subfamilies of cytochrome P450, such as 1A and 2B, are expressed in the human cervix (21). Involvement of other enzymes, such as lipoxynegenases (LOX) (22) and cyclooxygenases (COX) (23), in oxidative metabolism of NNK has been also reported.

A second metabolic pathway in NNK metabolism is carboxyl reduction resulting in the formation of 4-(methylisotrosmino)-1-(3-pyridyl)-1-butanol (NNAL). Experiments with cultured human tissues and human liver and lung microsomes have pointed to carboxyl reduction as a major metabolic pathway (24,25). The enzyme systems responsible for NNAL formation are not as well characterized as those for α-hydroxylation. The only enzyme identified to date in mouse hepatic and pulmonary microsomes capable of converting NNK to NNAL is 11β-hydroxysteroid dehydrogenase (11β-HSD) (26,27). NNAL is equally potent as NNK as a pulmonary and pancreatic carcinogen in F344 rats (28). Recent studies by Upadhyaya et al. have demonstrated that in A/J mouse the (S)-enantiomer of NNAL is significantly more potent as a lung tumorigen than the (R)-enantiomer (29).

In this study, we show that human epithelial cervical cells and subcellular fractions, namely microsomes and cytosol, are capable of metabolizing NNK by both oxidative and reductive pathways. We also investigated the possible role of 11β-HSD in NNAL formation and the enantiomeric composition of NNAL formed in these preparations. (R)-NNAL was formed as the main product in incubations with cells and microsomes and (S)-NNAL was the major product formed in incubations with cytosol.

Materials and methods

Chemicals

[5-3H]NNK (sp. act. 3.41 Ci/mmol; purity >97%) was purchased from ChemSyn Laboratories (Lenexa, KS) and further purified by normal phase high performance liquid chromatograph (HPLC) before use (30). Unlabeled NNK and its metabolites were synthesized as reported previously (31–34). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP+, NADPH, NAD, glycercylic acid (75% pure), hydrocortisone (cortisol), zinc sulfate, dithiothreitol (DTT) and barium hydroxide were purchased from Sigma Chemical Co. (St Louis, MO). Cortisone was purchased from Fluka Chemical Corp. (Milwaukee, WI) and phenylmethylsulfonyl fluoride (PMSF) and metyrapone from Aldrich Chemical Co. (Milwaukee, WI). [1,2,6,7-3H]Cortisol (sp. act. 62.0 Ci/mmol) was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Keratinocyte growth medium (KGM) was obtained from Clonetics (San Diego, CA), trypsin–EDTA from Life Technologies, Inc. (Germantown, MD), and phosphate-buffered saline (PBS) from Bio-Whittaker (Walkersville, MD).

Metabolism of NNK by human cervical cells

Normal human ectocervical and endocervical cell lines were established as previously described (35) from tissues obtained from hysterectomy specimens at the University of Chicago. Cells were maintained in Clonetics KGM and cultured in 5% CO2 at 37°C. For metabolism studies, cells were plated in T75 flasks and grown to ~70% confluence. One endocervical and two ectocervical cell lines were used in the metabolism study after either the third or fourth passage. Each cell line was derived from a different individual.

Ectocervical and endocervical cell cultures were grown as described above. Fresh medium was added 24 hours before the experiment. Immediately before the metabolism studies, the cells were harvested, pelleted, resuspended in PBS containing calcium and magnesium, and counted. [5-3H]NNK dissolved in water was added to give a final concentration of 5 pM NNK (sp. act. 0.8 Ci/mmol) in 0.5 ml. The number of cells per incubation ranged from 3.8 × 10^6 to 1 × 10^7/ml. Incubations were carried out at 37°C for 4 h. Cells were then pelleted and the supernatant was filtered through Gelman 0.45 μm Acrodiscs and stored at ~80°C until HPLC analysis. Incubations without cells using NNK alone served as controls.

Table I. Kinetic studies of NNAL and keto acid formation in human cervical microsomes incubated with NNK

<table>
<thead>
<tr>
<th>[NNK], μM (sp. act., μCi/μmol)</th>
<th>NNAL (pmol/mg/min)</th>
<th>Keto acid (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (1400)</td>
<td>13.0 ± 0.90 (n = 10)</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>50 (94.4)</td>
<td>115 ± 6.53 (n = 9)</td>
<td>4.9 ± 0.79</td>
</tr>
<tr>
<td>240 (21.3)</td>
<td>339 ± 24.2 (n = 9)</td>
<td>12.2 ± 3.0</td>
</tr>
<tr>
<td>330 (15.3)</td>
<td>472 ± 27.1 (n = 9)</td>
<td>24.4 ± 5.7</td>
</tr>
<tr>
<td>1200 (31.1)</td>
<td>858 ± 47.8 (n = 11)</td>
<td>34.9 ± 9.1</td>
</tr>
<tr>
<td>1640 (3.07)</td>
<td>949 ± 96.1 (n = 10)</td>
<td>78.7 ± 18.0</td>
</tr>
<tr>
<td>2570 (1.87)</td>
<td>1070 ± 125 (n = 9)</td>
<td>204 ± 68.0</td>
</tr>
<tr>
<td>6000 (0.83)</td>
<td>1280 ± 231 (n = 11)</td>
<td>291 ± 60.0</td>
</tr>
</tbody>
</table>

Metabolism of NNK by human cervical microsomal and cytosolic fractions

Preparation of microsomes and cytosol.

Cervical tissue samples from the uteri of women undergoing hysterectomy for non-malignant conditions were obtained from the University of Chicago and stored at ~80°C. Subcellular fractions were isolated by the following procedure. The tissue was homogenized in 0.25 M potassium phosphate buffer, pH 7.25, containing 0.15 M potassium chloride, 0.25 mM PMSF and 10 mM EDTA. The homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was then filtered through glass wool and centrifuged at 105,000 × g for 90 min; the cytosolic supernatant was carefully removed and stored at ~80°C. The microsomal pellet was dispersed using a glass homogenizer in sodium pyrophosphate buffer (140 mM, pH 7.25, containing 10 mM PMSF). Following centrifugation at 105,000 × g for 60 min, the supernatant was removed and the microsomal pellet was dispersed in 1–2 ml of 0.1 M potassium phosphate buffer, pH 7.25, containing 20% glycerol by volume, 10 mM EDTA, 0.25 mM PMSF and 0.1 mM DTT. For the kinetic studies, microsomes were isolated from 14 tissues pooled into a single sample. The isolation procedure was the same as that described except that the two high-speed centrifugations were carried out for 140 and 130 min, respectively. All microsomal preparations were stored at ~80°C.

The protein concentrations in all the microsomal and cytosolic fractions were determined using the Pierce Coomassie® Plus protein assay reagent and bovine serum albumin standards (Pierce Chemical Co., Rockford, IL) according to the manufacturer’s instructions. Absorbances were measured on a Beckman DU 640 spectrophotometer at 595 nm (Beckman–Coulter, Fullerton, CA).

Incubation of cervical microsomes and cytosol with NNK.

All incubations were done with 5 μM [5-3H]NNK. This concentration was selected based on previously published studies on the metabolism of NNK with human hepatic and lung microsomes (22,25,36). Incubations were performed at 37°C in potassium phosphate buffer, pH 7.4, using an NADPH-regenerating system. This system consisted of 1 mM NADP+, 5 mM glucose 6-phosphate, 3 mM MgCl2 and 0.8 or 1.6 units/ml of glucose-6-phosphate dehydrogenase. For the microsomal assays, protein concentrations ranged from 0.25 to 1 mg/ml. For the cytosolic assays, protein concentrations were 1.0 or 2.0 mg/ml. Incubation times were 60 min. Reactions were terminated by addition of 0.3 N ZnSO4 and 0.3 N NaOH or by placing the sample in boiling water for 1.5–2 min, cooling and then adding the ZnSO4 and Ba(OH)2. Samples were subsequently centrifuged at 13 000 r.p.m. (Heraeus centrifuge; Heraeus–Christ GmbH, Osterode, Germany) and the supernatant was filtered through a 0.45 μm Gelman syringe filter. All samples were stored at ~80°C until HPLC analysis. Boiled microsomes and boiled cytosol served as controls.

Kinetic studies of NNAL and keto acid formation

Cervical microsomes from the combined tissue sample were incubated with [5-3H]NNK diluted with cold NNK, at the concentrations and specific activities listed in Table I. Each incubation mixture consisted of 5 or 8 mM NADPH in 100 mM potassium phosphate buffer, pH 7.4, containing 3 mM MgCl2. The protein concentration was 0.31 mg/ml in a final volume of 0.5 ml. Multiple assays were conducted at each concentration. The data were analyzed and the kinetic parameters, Km and Vmax, were determined using GraphPad Prism software (GraphPad Inc., San Diego, CA).

Incubation of NNK with 11β-HSD inhibitors

Microsomal samples from two individuals were incubated with 5 μM [5-3H]NNK (sp. act. 1.0 Ci/mmol) and either cortisone or glycyrrhetic acid. Cortisone was dissolved in ethanol (10% v/v) or dimethylsulfoxide (DMSO) (2% v/v). Glycyrrhetic acid was dissolved in water. The final concentration of each inhibitor was 1 mM. Incubations were carried out at 37°C for 1 h.
NNK metabolism by human cervical tissue

using the NADPH-regenerating system described above. To determine the solvent effect on the formation of NNAL, incubations with either ethanol or DMSO alone were also performed. Incubations with microsomes isolated from the combined cervical tissue samples with 5 μM [5-3H]NNK and either glycyrhizic acid or metapyrone dissolved in water were also carried out. In the glycyrhizic acid study the concentration of inhibitor was 1 mM and the specific activity of NNK was 1.6 Ci/mmole. Incubation times were 60 min and the standard NADPH-regenerating system was used. In the metapyrone studies the concentration of inhibitor was 1 or 10 mM. The specific activity of NNK was 1.2 Ci/mmole. NADPH was used at a concentration of 10 mM. The protein concentration in all these incubations was 0.31 mg/ml. All reactions were terminated by placing the sample in boiling water and worked up as described above. Statistical analysis was conducted using Microsoft Excel software.

Unlabelled cortisol was purified by HPLC collection using a Phenomenex OptiSipil 10 ODS, 500 × 9.4 mm column with an isocratic flow of 47:53 (v/v) water/methanol at 4 ml/min. The elution of cortisol was monitored by UV absorption at 254 nm. After evaporation of solvents, the purified compound was stored at −20°C until used. Radioactive cortisol was purified using a Phenomenex C-18 Bondclone column, 250 × 7.8 mm under the same conditions used for the unlabelled compound. After collection, the solvent was partially evaporated to remove methanol and the remaining aqueous portion was extracted with ethyl acetate. The ethyl acetate extract was dried over sodium sulfate, filtered, and stored at −80°C until used.

Microsomes prepared from the combined tissue sample (0.31 mg/ml) were incubated in 100 mM potassium phosphate buffer, pH 7.4, for 1 h at 37°C with 0.1 mM [1,2,6,7-3H]cortisal (sp. act. 62.0 Ci/mmol) or 5 mM [1,2,6,7-3H]cortisol (sp. act. 1.2 Ci/mmol). Incubation times were 1 h and the volumes were 0.5 ml. Magnesium chloride and NADP+ were added and the solution was pre-incubated at 37°C for 5 min. It was determined that this procedure allowed >99% of the radioactive material to be delivered into the incubation mixture. Microsomes were then added and the incubation was initiated. Control incubations were conducted with boiled microsomes.

Incubations were terminated after 1 h by extracting the reaction mixture three times with 0.5 ml of ethyl acetate. Over 99% of the radioactivity was recovered in this manner. The ethyl acetate extract was dried over sodium sulfate, filtered, evaporated to dryness and reconstituted in 0.5 ml of 10% DMSO in water before HPLC analysis.

Cortisone formation was determined by HPLC analysis using a C-18 Bondclone column (10 μm, 5 mm × 300 mm; Phenomenex, Torrance, CA). Analytes were eluted using isocratic conditions with 47:53 (v/v) water/methanol (flow rate 1 ml/min). Under these conditions the approximate retention times were 11.5 and 13.5 min for cortisone and cortisol, respectively. Cortisone was identified by co-elution with a UV standard monitored at 254 nm.

HPLC analysis of NNK metabolites

All analyses involving radiolabeled NNK were carried out using a Phenomenex C-18 Bondclone column (10 μm, 5 mm × 300 mm). Solvent A was 20 mM sodium phosphate buffer, pH 7.4, while solvent B was methanol. The initial solvent composition was 100% solvent A. Methanol was added at a rate of 0.5%/min per minute for 60 min, by which time NNK and its metabolites had eluted. The flow rate was 1 ml/min. Radioactivity was detected using a β-Ram radiolow detector (INUS Systems, Tampa, FL). Each sample was spiked with UV standards and the radioactive peaks were identified based on co-chromatography with synthetic standards. The limit of detection for radioactive peaks was 0.1 nCi.

Determination of the stereochemistry of NNAL formed in incubations of NNK with cervical cells, cytosol and microsomes

The stereochemistry of NNAL was determined according to a published method with a modification of the HPLC system used for that analysis (37). Briefly, NNAL, formed during incubations with human cervical cells, microsomes or cytosol was collected by HPLC and, after partial evaporation of solvent, extracted five times with an equal volume of chloroform. The organic solvent was evaporated and the residue was reconstituted in benzene and derivatized overnight at 70°C with (S)-(−)-methylbenzyl isocyanate (MBIC) in the presence of triethylamine. The reaction mixture was then filtered, the benzene was evaporated and the residue was suspended in sodium phosphate buffer, pH 4.0, filtered and analyzed by HPLC. In contrast to the normal-phase HPLC analysis described in the original publication (37), the separation of the resulting derivatives was accomplished on a Rainin Microsorb-MV C-18 column, 5 μm particle size (Varian Associates, Inc., Woburn, MA). The initial solvent composition was 100% solvent A (2 mM sodium phosphate buffer, pH 7.0). Acetonitrile (solvent B) was added to reach a concentration of 45% in 90 min, by which time the derivatized NNAL enantiomers had eluted. The flow rate was 1 ml/min. Under these chromatographic conditions, the (S)-MBIC derivatives of R(-)-NNAL and S(+)-NNAL eluted at approximately 78 and 79 min, respectively (see Results). The order of elution was determined by reacting pure (S)-NNAL with (S)-MBIC followed by HPLC analysis.

Conformation of the identity of keto acid

Microsomes and cytosol. The keto acid identity was confirmed by its conversion to hydroxy acid, followed by derivatization to its methyl ester and co-elution with a UV standard upon HPLC analysis (38). Briefly, keto acid from microsomal or cytosolic incubations was collected under standard HPLC conditions. The solvent was partially evaporated and made alkaline with 8 N KOH, and 2.6 μmol of NaB₄H₆ in water was added. After 2 h, the solvent was evaporated and the residue was dissolved in 2 ml of methanol, 20 μl of H₂SO₄ was then added. The reaction mixture was heated at 50°C overnight. The resulting methyl ester of hydroxy acid was analyzed as previously described (38).

Cells. The identity of the keto acid formed during incubations with ectocervical cells was confirmed by collecting it under standard HPLC conditions and subsequent HPLC analysis using 60 mM acebuffer, pH 4.0. Analyses were performed using a Phenomenex C-18 Bondclone, 3.6 × 300 mm, 10 μm column. The initial solvent was 60 mM sodium acetate buffer, pH 4.0. Methanol was added at a rate of 0.5%/min at a flow rate of 1 ml/min. Under these conditions the keto acid elutes after 36 min, but under standard assay conditions it elutes after 21 min.

Results

Metabolism of NNK by human cervical cells

Representative HPLC traces of the incubations of NNK with human cervical cells are shown in Figure 1A (control), B and C. Human cervical epithelial cells metabolized NNK by both oxidative and reductive pathways (Figure 1B and C). In two lines of ectocervical cells the reductive metabolite of NNK, NNAL, eluting as two peaks representing E and Z isomers, was detected at 35.5 pmol/10⁶ cells (n = 2) and 16.9 ± 2.1 pmol/10⁶ cells (n = 3) (mean ± SD), respectively. These are approximately 0.7% and 0.34% of the initial amount of NNK. In these incubations the oxidative metabolite, keto acid, was found at concentrations similar to those of NNAL (Figure 2). The values for keto acid were 23.1 pmol/10⁶ cells (n = 2) and 8.4 ± 0.97 pmol/10⁶ cells (n = 3) (0.47% and 0.17% of the initial amounts of NNK, respectively). The amount of NNAL detected in the incubation with endocervical cells was 6.2 ± 0.36 pmol/10⁶ cells (0.12% of the initial amount of NNK). The corresponding value for the keto acid was 4.5 ± 1.2 pmol/10⁶ cells (0.09% of the initial amount of NNK). One ectocervical cell line seemed to be capable of converting NNK to the keto alcohol (3.5 pmol/10⁶ cells; Figure 1B). The identity of the keto alcohol was confirmed by co-elution with a UV standard. In addition, two possible metabolites of NNK of unknown identity, labeled X and Y, eluting at 37 and 67 min, respectively, were detected in this incubation with ectocervical cells (Figure 1B).

Metabolism of NNK by human cervical microsomes and cytosol

Incubations were performed with microsomes isolated from nine individuals. In all incubations the major metabolite of NNK was NNAL (Figure 1D). Its rate of formation ranged from 1.88 ± 0.19 (mean ± SD) to 4.95 pmol/min/mg (Figure 3). Cytosolic fractions were obtained from four individual tissues from which microsomes were also isolated. Boiled cytosol served as a control (Figure 1E). Similar to the microsomal incubations, NNAL was the major product detected (Figure 1F). The rate of NNAL formation in the cytosolic incubations ranged from 1.44 ± 0.56 to 2.08 ± 0.14 pmol/mg/min. Under the experimental conditions used in this study, approximately 2.3–5.9% and 1.7–2.5% of the initial amounts
of NNK were metabolized to NNAL by human cervical microsomes and cytosol, respectively.

Keto acid was detected in three of nine incubations of NNK with microsomal preparations and in all four incubations conducted with cytosol (Figure 3). Its rate of formation in microsomal incubations ranged from not detectable to
NNK metabolism by human cervical tissue

Fig. 4. HPLC analyses of NNAL diastereomers. NNAL obtained from incubations of 5 µM [5-3H]NNK with either human cervical cells, cytosol or microsomes was derivatized with (S)–(−)-MBIC. (A) synthetic, racemic unlabeled NNAL, (B) incubation with human ectocervical cells #1, (C) incubation with human ectocervical cells #2, (D) incubation with human cervical cytosol and (E) incubation with human cervical microsomes.

0.13 ± 0.05 pmol/mg/min (mean ± SD). In incubations with cytosol, the rate of keto acid formation was 0.008–0.033 ± 0.009 pmol/mg/min. Similar to the incubations with ectocervical cells, keto alcohol was also detected in some of the cytosolic and microsomal incubations with NNK (Figure 1D and F).

Determination of the stereochemistry of NNAL formed by human cervical cells and subcellular fractions

Upon derivatization with (S)-MBIC, we found that NNAL formed in incubations with either ectocervical or endocervical cells, or microsomes, was almost exclusively the (R)-enantiomer while the (S)-enantiomer of NNAL was predominantly formed in incubations with cervical cytosol (Figure 4).

Kinetic studies of NNAL and keto acid formation

The kinetics of NNAL formation from NNK were determined by incubating [5-3H]NNK at the concentrations listed in Table I under conditions described in Materials and methods. All experiments were carried out under conditions that resulted in a linear response with respect to time and protein concentration (data not shown).

Parameters of Michaelis–Menten kinetics were determined for the reduction of NNK to NNAL (Figure 5A) and the formation of keto acid (Figure 5B) using substrate concentrations ranging from 5 µM to 6 mM. The apparent $K_m$ for NNAL formation was 739 µM and the $V_{max}$ was 1395 pmol/mg/min; the apparent $K_m$ for keto acid was 7075 µM and the $V_{max}$ was 650 pmol/mg/min.

Inhibition of NNAL formation by substrates of 11β-HSD

In order to determine whether 11β-HSD is involved in the carbonyl reduction of NNK in human cervical tissue, we conducted experiments to determine the degree of inhibition of NNAL formation by inhibitors of 11β-HSD. The inhibition of NNAL formation by cortisone in incubations of NNK with human cervical microsomes is summarized in Figure 6. The data demonstrate that both ethanol and DMSO (used as vehicles to dissolve cortisone) inhibit the formation of NNAL. Therefore, incubations with glycyrrhizic acid and metyrapone were performed, since both compounds are water-soluble and are also inhibitors for 11β-HSD. The inhibition of NNAL formation by glycyrrhizic acid and metyrapone ranged from 16% to 32% and from 33% to 80%, respectively (Figure 6). The levels of NNAL formed in these incubations with inhibitors were significantly lower than the levels of NNAL determined in controls ($P < 0.01$).

Incubations of cervical microsomes with cortisol

In order to determine the extent of involvement of 11β-HSD in the reduction of NNK to NNAL, incubations with cortisol were carried out as described in Materials and methods. Incubations of cortisol (0.1 µM) with human cervical microsomes yielded 0.0044 pmol/mg/min of cortisone. Incubations with cortisol at a higher concentration (5.0 µM) yielded 0.41 pmol/mg/min of cortisone.
Discussion

To our knowledge, this is the first study addressing the capacity of human cervical tissue to metabolize NNK, a tobacco-specific carcinogen previously detected in human cervical mucus (18). The results clearly demonstrate that human cervical tissue is capable of metabolizing NNK by both $\alpha$-hydroxylation and reductive pathways, as is evident from the formation of keto acid and NNAL, respectively. NNAL was formed as the major metabolite in all incubations of NNK with human cervical cells, microsomes and cytosol. The stereochemical isomer of NNAL formed by cervical cells and microsomes differed from that formed by cytosol.

Oxidation of NNK is catalyzed by multiple forms of cytochrome P450, a family of hemoproteins involved in the metabolism of various xenobiotics including carcinogens, drugs and environmental pollutants (20). Studies by Smith et al. (25) and Staretz et al. (36) showed that in human liver P450 2A6 and 3A4 isozymes are responsible for $\alpha$-methylene hydroxylation of NNK, while $\alpha$-methyl hydroxylation is predominantly catalyzed by P450 1A2. In human lung, $\alpha$-methyl hydroxylation of NNK is probably catalyzed by P450 2A6 (25). Subfamilies of P450 1A and 2B have been reported to be expressed in the human cervix (21), indicating that human cervical tissue may be capable of metabolizing NNK via an oxidative pathway. This has been confirmed in the present study.

The levels of $\alpha$-hydroxylation products detected in microsomal incubations in our study were low relative to those of NNAL. This is consistent with results of NNK metabolism by various human tissue and microsomal preparations previously reported (22,24,25). In contrast, in incubations of NNK with human cervical cells, the levels of products resulting from NNK oxidation were similar to the levels of NNAL. This may be because these cells represent a population of pure epithelial cells, whereas the microsomal preparations contain both epithelial and stromal cells. These results suggest that cervical epithelial cells, where cervical cancer usually originates (39), may have greater potential for oxidative metabolism of NNK than was indicated by the metabolism studies conducted with the whole tissue preparations, namely microsomes and cytosol.

We found that the cytosolic fraction isolated from human cervical tissue was also capable of metabolizing NNK by oxidation. Similar to microsomes, the levels of oxidative products formed in cytosolic incubations were low relative to the levels of NNAL. As far as we know, no cytosolic enzymes capable of $\alpha$-hydroxylation of NNK have been reported. Therefore, detection of these products in cytosolic incubations was unexpected, although Hecker et al. (40) did report low levels of oxidative products formed upon incubation of N-nitrosopyrrolidine with rat liver cytosol. It has been well documented that LOX and COX are present in the cytosolic fraction (41,42). The involvement of these enzymes in the microsomal metabolic activation of NNK, probably by a free-radical mechanism, has been shown (22,23). Thus, these enzymes could be involved in the metabolic activation of NNK by human cervical cytosol.

In this study we did not attempt to characterize the microsomal or cytosolic enzymes responsible for oxidation of NNK. The presence of $\alpha$-hydroxylation products in incubations with cervical microsomes, however, clearly demonstrates that human cervical tissue can metabolize NNK via pathways that produce DNA-damaging intermediates. The characterization of the enzymes responsible for the oxidation of NNK by cervical subcellular fractions and elucidation of the mechanisms involved is an important area for future studies.

The reductive metabolite of NNK, NNAL, may play an important role in the carcinogenicity of NNK. Several laboratory studies have demonstrated that its carcinogenic potency is similar to that of NNK (20,28,43). It has recently been shown that the stereochemistry of NNAL may be an important factor in the carcinogenicity of NNK (29). The (S)-enantiomer of NNAL was a more potent lung tumorigen in the A/J mouse than the (R)-NNAL enantiomer (29). Its potency was similar to that of NNK. In the present study we found that incubation of NNK with human cervical cells and microsomes resulted in the predominant formation of the (R)-NNAL, while (S)-
NNAL was formed almost exclusively in incubations with cervical cytosol. This result indicates that in the human cervix, several enzymes, in this case associated with different subcellular fractions, may be involved in the carbonyl reduction of NNK.

The enzymes responsible for the microsomal carbonyl reduction of NNK are not as well characterized as those involved in its oxidation. Recently Maser et al. (26,27) identified 11β-HSD, an enzyme involved in the oxidoreduction of glucocorticoids, as being responsible for the carbonyl reduction of NNK to NNAL in mouse liver and lung microsomes. This enzyme is distributed in many tissues and has at least two isoforms (44,45). 11β-HSD1 is an NADPH-dependent reductase that has both reductive and oxidative properties in vitro. 11β-HSD2 is an NAD-dependent form functioning solely as a dehydrogenase. The inhibition of NNAL formation by cortisone, glycyrrhizic acid and metyrapone, all substrates for 11β-HSD, indicates a possible role of this enzyme in carbonyl reduction of NNK in human cervical microsomes. However, incubation of human cervical microsomes with 5 mM cortisol resulted in a rate of formation of cortisone of 0.41 pmol/mg/min. The rate of formation of cortisone determined in the incubation with mouse liver microsomes under identical conditions was 303 pmol/mg/min (data not shown). When 5 mM NNK was incubated with microsomes obtained from the combined cervical tissue sample under conditions identical to those used for the cortisol incubations, a rate of NNAL formation of 13 pmol/mg/min was determined (data from the kinetic studies). This rate of NNAL formation is ~30-fold greater than the rate of cortisol formation determined in the cervical microsomal sample. Collectively, these results suggest that the enzymes responsible for carbonyl reduction of NNK in human cervical microsomes have a greater affinity for NNK than for cortisol. This would not be expected if the primary enzyme responsible for NNAL formation were 11β-HSD. In addition, 10 mM metyrapone, which represents a 2000-fold excess of inhibitor, did not completely eliminate the formation of NNAL (~80% inhibition was recorded), so it appears that enzymes other than 11β-HSD are also involved in carbonyl reduction of NNK in human cervical microsomes.

The role of NNAL in the carcinogenicity of NNK is not well established. NNAL can be converted to its glucuronide conjugate, which can be eliminated and, therefore, may play a role in carcinogen detoxification. Diastereromic glucuronides of NNAL have been identified in patas monkey urine and, along with unconjugated NNAL, have been detected in smokers’ urine (46,47). Maser et al. have suggested that the NNK/NNAL equilibrium in a given tissue is extremely important for organ susceptibility to the development of cancer (25). Thus, the high level of NNAL formed by rat liver facilitates elimination of NNK, consequently resulting in low tumor yield in this organ, while the lack of NNAL formation in rat nasal mucosa leads to high tumor incidence in this organ. In our study, we determined a Vmax/Km ratio (intrinsic clearance) of 1.95 for NNAL in one microsomal preparation. This is similar to that observed in human lung, 0.58 (25), patas monkey lung microsomes, 0.53 (48) and mouse lung microsomes, 1.04 (27), but differs from that recently reported in human lung microsomes, 7.3–43.9 (49). The last study indicates that there is large variability between individuals in the intrinsic clearance of NNAL.

In summary, we have demonstrated that the tobacco-specific carcinogen, NNK, is metabolized by human cervical tissue to genotoxic and carcinogenic products. This study, together with our previous investigations (18,19,50), further supports the epidemiologic findings that associate cigarette smoking as a factor in the etiology of cervical cancer. Further studies are in progress to develop a suitable animal model to determine the roles of NNK, NNAL and HPV individually and in combination, in the development of cervical cancer.

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References


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