Formation and Distribution of NNK Metabolites in an Isolated Perfused Rat Lung

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ABSTRACT:

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a lung-specific tobacco carcinogen. Metabolism is critical to its elimination given its lipophilic nature. Although NNK can be metabolized through detoxification pathways that safely eliminate it from the body, it can also be bioactivated, resulting in the formation of potentially carcinogenic DNA adducts. The isolated perfused rat lung (IPRL) system was used to determine the effect of NNK perfusate concentration (0.1 and 1.2 μM) on the formation and distribution of metabolites, the level of individual DNA adducts, and total covalent binding in the lung. Coadministration of the chemopreventive agent phenethyl isothiocyanate (PEITC; 20 μM) was also examined to determine its effect on NNK metabolism. NNK was readily metabolized in the IPRL system. In the 0.1 μM perfusions approximately 55% of metabolites formed were through detoxification pathways, whereas roughly 30% were the result of bioactivation pathways. An increase in NNK concentration in-

creased the percentage of unmetabolized NNK and decreased the apparent metabolic clearance in the lung, but the metabolite profiles remained similar between concentrations. The addition of PEITC reduced the formation of oxidative metabolites and increased 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) formation and the percentage of unmetabolized NNK. PEITC also significantly decreased the formation of DNA adducts in the lung tissue. The level of O^2 -[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O^2 -POB-dThd) and O^6 -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O^6 -POB-dGuo) decreased by 70 to 75%, and that of O^6 -methylguanine (O^6 -methyl-Gua) and 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua) decreased by 40 to 45%. Pyridylhydroxybutyl-DNA adducts were not detected in any of the treatment groups. Thus, the IPRL system is useful in determining pulmonary metabolism and DNA adduct formation separate from other metabolizing organs.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK] is a tobacco-specific carcinogen. It has been established as a strong carcinogen in rodents (Hecht, 1998) and has been classified as a human carcinogen by the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2007). One of the most intriguing characteristics of NNK is its ability to selectively induce pulmonary tumors in rodents (Hecht, 1998). This lung specificity, along with biochemical and epidemiologic data, implicates NNK as a plausible contributor to the development of lung cancer in smokers (Hecht, 2003; IARC Working Group

on the Evaluation of Carcinogenic Risks to Humans, 2007; Yuan et al., 2009).

The carcinogenic effects of NNK are primarily attributed to its metabolic activation. NNK is extensively metabolized in rodents and humans, which is illustrated by the low levels of NNK excreted unchanged in the urine (Hecht, 1998). An important metabolic pathway of NNK is carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a carcinogenic metabolite that undergoes subsequent metabolism through pathways parallel to those of NNK (Fig. 1). Both NNK and NNAL are detoxified via *N*-oxidation to form their respective *N*-oxide metabolites, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK-*N*-oxide) and 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol (NNAL-*N*-oxide). Likewise, both compounds undergo bioactivation through their α -hydroxylation pathways, resulting in the formation of DNA adducts. α -Methylene hydroxylation of NNK and NNAL results in the formation of the reactive intermediate methanediazohydroxide, which can form methyl DNA adducts. This pathway ultimately results in the formation of the metabolites 4-oxo-4-(3-pyri-

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ABBREVIATIONS: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; NNAL-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol; keto acid, 4-oxo-4-(3-pyridyl)butyric acid; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butyric acid; keto alcohol, 4-oxo-4-(3-pyridyl)butanol; POB, pyridyloxobutyl; PHB, pyridylhydroxybutyl; diol, 4-hydroxy-4-(3-pyridyl)butanol; O^6 -POB-dGuo, O^6 -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine; O^2 -POB-dThd, O^2 -[4-(3-pyridyl)-4-oxobut-1-yl]thymidine; 7-POB-Gua, 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine; O^6 -PHB-dGuo, O^6 -[4-(3-pyridyl)-4-hydroxybut-1-yl]-2'-deoxyguanosine; O^2 -PHB-dThd, O^2 -[4-(3-pyridyl)-4-hydroxybut-1-yl]thymidine; 7-PHB-Gua, 7-[4-(3-pyridyl)-4-hydroxybut-1-yl]guanine; O^6 -methyl-Gua, O^6 -methylguanine; PEITC, phenethyl isothiocyanate; IPRL, isolated perfused rat lung; HPLC, high-pressure liquid chromatography; LSC, liquid scintillation counting; AUC, area under the curve; CL_{app} , apparent clearance; ER_{app} , apparent extraction ratio; $t_{1/2, \text{app}}$, apparent half-life; P450, cytochrome P450.

FIG. 1. A simplified metabolic scheme of NNK and NNAL. *, represents the location of the tritium label. A more detailed metabolic scheme has been previously published (Hecht, 1998).

dyl)butyric acid (keto acid) and 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid). When NNK undergoes α -methyl hydroxylation, the unstable intermediate 4-(3-pyridyl)-4-oxobutanediazohydroxide is formed, which can react with water to yield the metabolite 4-oxo-4-(3-pyridyl)butanol (keto alcohol) or bind to DNA to form pyridyloxobutyl (POB)-DNA adducts. Likewise, the α -methyl hydroxylation of NNAL can produce pyridylhydroxybutyl (PHB)-DNA adducts or the metabolite 4-hydroxy-4-(3-pyridyl)butanol (diol). Previous *in vivo* studies in which NNK and NNAL were chronically administered in the drinking water of rats showed that the POB and PHB-DNA adducts that developed in the lung included O⁶-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O⁶-POB-dGuo), O²-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O²-POB-dThd), 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua), O⁶-[4-(3-pyridyl)-4-hydroxybut-1-yl]-2'-deoxyguanosine (O⁶-PHB-dGuo), O²-[4-(3-pyridyl)-4-hydroxybut-1-yl]thymidine (O²-PHB-dThd), and 7-[4-(3-pyridyl)-4-hydroxybut-1-yl]guanine (7-PHB-Gua) (Lao et al., 2006, 2007; Upadhyaya et al., 2008). The formation of the methyl adduct O⁶-methylguanine (O⁶-methyl-Gua) after exposure to NNK was also observed (Upadhyaya et al., 2009), which is consistent with previous studies (Belinsky et al., 1986, 1991; Devereux et al., 1991; Hecht, 1998).

The lung tumorigenicity of NNK in rats is inhibited by phenethyl isothiocyanate [PEITC] (Morse et al., 1989; Hecht et al., 1996). PEITC is found as a conjugate in certain cruciferous vegetables, such as watercress. *In vitro* and *in vivo* data indicate that PEITC inhibits the oxidative metabolism of NNK, resulting in a decrease in DNA adduct formation in the lung, thus inhibiting the carcinogenic effects of NNK

(Morse et al., 1989; Staretz and Hecht, 1995; Hecht et al., 1996; Staretz et al., 1997a,b).

Because metabolic activation seems to be crucial to lung tumor formation, it is necessary to examine the pulmonary metabolism of NNK separately from other metabolizing organs, such as the liver. The isolated perfused rat lung system (IPRL) maintains the structural integrity of the lung, potentially providing a system more representative of localized *in vivo* metabolism than other *in vitro* systems. The purpose of this study was to better understand the carcinogenicity of NNK in the lung by examining the effects of PEITC and NNK concentration on perfusate and tissue metabolites, as well as DNA adduct formation in the IPRL system. The present lung perfusions expand on previous studies by quantitating metabolites in the tissue and perfusate, allowing for a better understanding of the distribution of metabolites between the perfusate and tissue. Furthermore, the characterization of individual DNA adducts shows that metabolic bioactivation in lung leads directly to DNA damage, without the contribution of the liver. This is the first study to our knowledge to examine the formation of DNA adducts and the effect of PEITC on NNK metabolism in the lung using the IPRL system.

Materials and Methods

Chemicals. Unlabeled NNK, racemic NNAL, NNK-*N*-oxide, NNAL-*N*-oxide, keto alcohol, keto acid, and diol were purchased from Toronto Research Chemicals (North York, ON, Canada). Hydroxy acid was synthesized as previously reported (McKennis et al., 1964). [5-³H]NNK (21.7 Ci/mmol) was purchased from Moravsek Biochemicals (Brea, CA). High-pressure liquid chro-

matography (HPLC) grade acetonitrile and isopropanol were obtained from Thermo Fisher Scientific (Hanover Park, IL). Dextran 70 was acquired from Pharmacosmos (Holbaek, Denmark). The Puregene DNA isolation kit was purchased from QIAGEN (Valencia, CA). PEITC, bovine serum albumin (A7906-500G), and all the other chemicals were acquired from Sigma-Aldrich (St. Louis, MO).

Animals. Male Fischer 344 rats were purchased from Charles River Breeding Laboratories (Portage, MI). They were housed two to three per cage containing corn cob bedding and were maintained under standard conditions ($20 \pm 2^\circ\text{C}$, 12-h light/dark cycle). Animals were given food (Teklad 2018; Harlan Teklad, Madison, WI) and water ad libitum. After arrival, they were allowed to acclimate in the housing facility for at least 1 week before use. The animal protocol for these experiments was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Isolated Lung Perfusion. The general principles of the isolated lung perfusion procedure used were adopted from the laboratory of Dr. Douglas Wangenstein (University of Minnesota) and have been described previously (Wangenstein et al., 1986, 1996). To remove the lungs from the chest cavity, rats were anesthetized with a 60-mg/kg intraperitoneal dose of pentobarbital sodium (Ovation Pharmaceuticals, Deerfield, IL). A tracheotomy was performed, and a 0.75- to 1-inch piece of PE 240 tubing (BD Biosciences, San Jose, CA) was inserted into the trachea and tied securely into place with suture (Deknatel, Mansfield, MA). Next, an incision was made into the abdomen, and the chest cavity was opened by cutting up the midline of the rib cage. The chest cavity was held open with a retractor, and 100 units of heparin sodium (APP Pharmaceuticals, Schaumburg, IL) was slowly injected into the right heart immediately below the pulmonary artery. A small incision was then made at the site of the heparin injection, and a polyethylene cannula (PE 190) connected to the perfusate supply was inserted into the pulmonary artery and tied into place. The heart was cut thoroughly to allow for unrestricted flow of perfusate out of the lung. The lungs were then excised from the chest cavity and suspended by the trachea from a ring stand, where they were rinsed of blood with perfusate. The lungs were inflated and deflated two to three times to help facilitate the removal of the blood from the vessels, after which they were inflated at a constant pressure of 4 cm H_2O . The flow rate was set to 8 ml/min, and once the lungs were adequately rinsed, as determined by visual inspection, they were placed in the jacketed beaker that served as the reservoir.

The lungs were perfused in a 50-ml recirculating system with a Ringer's solution, pH 7.4, that was composed of 2.68 mM KCl, 1.25 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.82 mM CaCl_2 , 5.55 mM D-glucose, 137 mM NaCl, 12 mM HEPES, 1% dextran 70, and 0.5% albumin. The perfusate was oxygenated with 95% O_2 and 5% CO_2 , and the system was maintained at 37°C . The pressure, temperature, and pH were monitored with transducers throughout the perfusion. A lung was discarded if excessive swelling and water retention were observed throughout the course of the perfusion.

Metabolism studies. The metabolism of NNK in the lung was examined using two initial concentrations of $[5\text{-}^3\text{H}]\text{NNK}$ with and without PEITC. Three animals were used for each of four treatments: 1) 0.1 μM NNK; 2) 0.1 μM NNK + 20 μM PEITC; 3) 1.2 μM NNK; and 4) 1.2 μM NNK + 20 μM PEITC. Once the lungs were rinsed of blood and a recirculating perfusion was established, a bolus dose of each of the appropriate compounds was injected into the perfusate reservoir. Perfusate samples of 250 μl were taken from the reservoir at 0, 1, 5, 10, 20, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min postdose. After the final sample was collected, the lungs were perfused with 50 ml of blank perfusate. The tissue was then minced, weighed, flash-frozen in liquid nitrogen, and stored at -80°C until further analysis.

DNA adduct studies. The perfusions for studying DNA adducts were carried out in the same manner as those conducted for metabolism, except that unlabeled NNK was used and perfusate samples were not collected. After the 180-min perfusion, the lungs were perfused with 50 ml of blank perfusate; the tissue was then minced, weighed, flash-frozen in liquid nitrogen, and stored at -80°C until analysis for individual DNA adduct formation was performed.

Perfusate Analysis. The amount of total radioactivity in each perfusate sample was determined by analyzing a 50- μl aliquot by liquid scintillation counting (LSC). The remaining 200 μl of each sample was used to quantitate metabolite formation. Protein was precipitated from the perfusate samples by adding 500 μl of acetonitrile, vortexing, and then centrifuging at 13,000g (235B; Thermo Fisher Scientific) for 3 min. The supernatant was then con-

centrated to dryness with a stream of nitrogen while heating in a 37°C water bath. The samples were reconstituted with 220 μl of 20 mM NaHPO_4 buffer containing 1 mM EDTA and centrifuged at 13,000g for 1 min. The metabolites were analyzed by injecting 100 μl of the final supernatant onto HPLC with UV and radioflow detectors.

Tissue Analysis: Metabolites and Total Covalent Binding. Tissue samples from the metabolism perfusions were used to quantitate the metabolites and to estimate the total covalent binding in the tissue. The lung samples were homogenized with a PowerGen 125 homogenizer (Thermo Fisher Scientific). A small aliquot of homogenate was solubilized with 6 ml of 1 M NaOH and analyzed by LSC to estimate the total radioactivity in the tissue. The remaining homogenate was used to quantitate the metabolites and covalent binding in the lung.

Metabolites. To the remaining homogenized lung tissue, 6 ml of 0.1 M HCl was added, and the tissue was further homogenized. The sample was then centrifuged at 2950g (Sorvall Legend RT; Thermo Fisher Scientific) for 30 min. The resulting tissue pellet was used for total covalent binding analysis. The resulting cloudy supernatant was used to determine tissue metabolites and was transferred to a clean test tube and neutralized. To buffer the solution, 1 ml of NaPO_4 (0.1 M) was added to the supernatant before neutralizing with 1 M NaOH. The neutralized supernatant was then centrifuged at 1228g (Thermo Fisher Scientific) for 20 min. The resulting clear supernatant was transferred and concentrated to dryness in a 37°C water bath with a stream of nitrogen. Once dried, the sample was reconstituted with 400 μl of distilled H_2O . Tissue metabolites were determined by injecting 100 μl of the reconstituted sample onto HPLC with UV and radioflow detectors.

Total covalent binding. The tissue pellet that remained after the initial metabolite extraction was used to estimate the total covalent binding in the lung tissue. The pellet was homogenized with 6-ml aliquots of HCl (0.1 M) and then centrifuged at 1228g for 15 min until the counts in the wash solution were not more than twice the level of background, approximately nine washes. The tissue pellet was then solubilized with 10 ml of NaOH (1 M) and analyzed by LSC. The protein concentration of the solubilized tissue pellet was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific) with an albumin standard.

HPLC Analysis. HPLC analysis was performed on a system that consisted of a Waters 600 system controller, two Waters 501 pumps, a Waters 440 absorbance detector (254 nm) (Waters, Milford, MA), a Hewlett Packard 1100 series autosampler (Palo Alto, CA), and a β -RAM radioflow detector (IN/US Systems, Tampa, FL). A Luna C18(2) reversed-phase column (250×4.6 mm, 5 μm ; Phenomenex, Torrance, CA) was used for the separation. The metabolites were eluted using a linear gradient from 92% A (20 mM NaHPO_4 and 1 mM EDTA, pH 6.0) to 80% A over 30 min, and then to 50% A in 5 min; B was acetonitrile (Jalas et al., 2003). The flow rate was 0.5 ml/min, which was mixed with 1.5 ml/min Monoflow (National Diagnostics, Atlanta, GA).

DNA Isolation. DNA was isolated using a slightly modified version of the Gentra Puregene Tissue Kit protocol (QIAGEN). A single lung was weighed and divided into approximately two 500-mg aliquots. The samples were kept frozen on dry ice and minced with a razor blade. The aliquots were transferred to a 15-ml glass homogenizer containing 5 ml of Cell Lysis Solution (QIAGEN) and were homogenized on ice. The homogenate was transferred to a 50-ml centrifuge tube into which the remaining 13 ml of Cell Lysis Solution was added. Then, 75 μl of Puregene Proteinase K (QIAGEN) was added, and the tubes were inverted 25 times before being placed in an orbital shaker (Maxq 4450; Thermo Fisher Scientific) and shaken at 80 rpm overnight at 55°C . Once the tissue was completely lysed, 90 μl of RNase A (QIAGEN) solution was added. The tubes were inverted 25 times and then replaced in the orbital shaker for 60 min at 80 rpm and 37°C . The solution was then put on ice for 3 to 5 min. Six milliliters of Protein Precipitation Solution (QIAGEN) was added, and the samples were vortexed on high for approximately 20 s. The samples were then centrifuged at 2000g (Sorvall Legend RT; Thermo Fisher Scientific) for 10 min. The resulting supernatant was carefully poured into a clean 50-ml tube containing 18 ml of ice-cold isopropanol (100%). The tube was slowly inverted 50 times, resulting in the formation of a clump of DNA strands. The DNA was rinsed three times with 6-ml aliquots of 70% ice-cold ethanol and then three more times with 6-ml aliquots of 100% ice-cold ethanol. The DNA was then dried with a gentle stream of nitrogen. Samples were stored at -4°C until further analysis.

Quantitation of DNA Adducts. DNA adduct formation was analyzed using the liquid chromatography/electrospray ionization-mass spectrometry/mass spectrometry-selected reaction monitoring method previously described for POB or PHB DNA adducts (Lao et al., 2006, 2007; Upadhyaya et al., 2008). In brief, deuterated POB or PHB internal standards were added to 0.5 to 2 mg of isolated DNA, and neutral thermal hydrolysis was carried out at 100°C for 30 min. Then, enzymatic hydrolysis was carried out with micrococcal nuclease, phosphodiesterase II (Worthington Biochemicals, Freehold, NJ), and alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN). The resulting hydrolysate was then purified by solid-phase extraction (Strata-X cartridge; Phenomenex) and injected into a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fisher Scientific). The limits of detection for the POB and PHB adducts were 7-POB-Gua, 3 fmol/mg DNA; O^6 -POB-dGuo, 1 fmol/mg DNA; O^2 -POB-dThd, 100 amol/mg DNA; 7-PHB-Gua, 18 fmol/mg DNA; O^6 -PHB-dGuo, 1.5 fmol/mg DNA; and O^2 -PHB-dThd, 1.5 fmol/mg DNA.

For O^6 -methyl-Gua analysis, 0.05 to 1 mg of each DNA sample plus [CD_3] O^6 -methyl-Gua internal standard was dissolved in 1 ml of HCl (0.1 N) and heated at 80°C for 30 min, cooled, and neutralized with 1 N NaOH to pH 7.0. The resulting solution was applied to a solid-phase extraction cartridge (Strata-X; Phenomenex) and analyzed by capillary liquid chromatography/electrospray ionization-mass spectrometry/mass spectrometry-selected reaction monitoring as described previously (Upadhyaya et al., 2009). The limit of detection for O^6 -methyl-Gua was 3 fmol/mg DNA.

Pharmacokinetic Analysis. The apparent pharmacokinetic parameters for the metabolism of NNK in the lung were estimated by noncompartmental analysis of the NNK concentration-time data in the perfusate using WinNonlin version 5.2 (Pharsight, Mountain View, CA). The area under the curve from 0 to 180 min (AUC_0^{180}) was calculated by WinNonlin via the linear trapezoidal rule. The AUC_{180}^{00} was extrapolated using the concentration of NNK in the

perfusate at 180 min (C_{last}) and the estimated elimination rate constant, λ_z . The concentration-time data from 45 min to 180 min were used to estimate λ_z , using uniform weighting. The combined value of AUCs yielded the AUC_0^{00} , which was used to calculate the apparent clearance (CL_{app}). The apparent extraction ratio (ER_{app}) was calculated by dividing CL_{app} by the perfusate flow rate (8 ml/min).

Statistical Analysis. Statistical analysis of the data was carried out using SigmaStat version 3.1 (Systat Software, Inc., San Jose, CA). Unpaired t tests were used to compare tissue metabolites, perfusate metabolite AUCs, total covalent binding, and individual DNA adduct levels between the PEITC and non-PEITC groups at the two doses. A two-way analysis of variance was used to compare the apparent pharmacokinetic parameters between the PEITC and non-PEITC groups at each dose, and to compare the parameters between the two doses of NNK. The Holm-Sidak method was used for pairwise multiple comparisons. A p value of <0.05 was considered significant for all the tests.

Results

0.1 μ M NNK. The perfusate concentration-time profiles of NNK and its metabolites in the 0.1 μ M NNK perfusions are shown in Fig. 2, A, C, and E. Figure 2A shows the concentration profile of NNK. Less than 5% of the final metabolites were attributed to unmetabolized NNK after the 180-min perfusion. The NNK concentration-time data were used to estimate the apparent pharmacokinetic parameters (Table 1). The CL_{app} was 1.07 ± 0.09 ml/min; the ER_{app} was 0.13 ± 0.01 ; and the apparent half-life ($t_{1/2,app}$) was 34 ± 5 min.

NNK-*N*-oxide was the major metabolite in the perfusate with a concentration of 33.8 ± 0.8 pmol/ml, which accounted for approximately 49% of the total metabolites in the perfusate. The final con-

FIG. 2. Concentration (picomoles per milliliter) $\pm$ S.D. versus time (minutes) profiles of NNK and metabolites in the perfusate of 0.1 μ M NNK (A, C, E) and 0.1 μ M NNK + PEITC (B, D, F) ($n = 3$). Symbol designations are as follows: NNK, $\bullet$; NNK-*N*-oxide, $\circ$; NNAL, $\blacksquare$; NNAL-*N*-oxide, $\square$; keto alcohol, $\blacktriangleleft$; keto acid, $\triangleleft$; diol, $\blacktriangle$. The profile of NNAL is replotted in E and F for comparison purposes. Where error bars are not apparent, they are smaller than the symbol.

TABLE 1

Apparent pharmacokinetic parameters (mean $\pm$ S.D., $n = 3$) for the metabolism of NNK in the lung

A two-way analysis of variance with Holm-Sidak was used for determining significant differences.

	0.1 μ M NNK	0.1 μ M + PEITC	1.2 μ M NNK	1.2 μ M + PEITC
$t_{1/2,app}$ (min)	34 $\pm$ 5	108 $\pm$ 8 ^a	64 $\pm$ 16 ^a	149 $\pm$ 11 ^b
V_z (ml)	51 $\pm$ 3	60 $\pm$ 1	61 $\pm$ 11	76 $\pm$ 3 ^b
CL_{app} (ml/min)	1.07 $\pm$ 0.09	0.39 $\pm$ 0.03 ^a	0.66 $\pm$ 0.09 ^a	0.36 $\pm$ 0.03 ^b
ER_{app}	0.13 $\pm$ 0.01	0.05 $\pm$ 0.01 ^a	0.08 $\pm$ 0.01 ^a	0.04 $\pm$ 0.00 ^b

^a $p < 0.05$ compared with 0.1 μ M NNK.

^b $p < 0.05$ compared with 1.2 μ M NNK.

FIG. 3. Concentration of metabolites (picomoles per milligram of tissue) $\pm$ S.D. in the tissue after 180-min perfusion ($n = 3$). Open bars represent 0.1 μ M NNK, and shaded bars are 0.1 μ M NNK + PEITC. *, $p < 0.05$.

TABLE 2

Levels of individual DNA adducts (fmol/mg DNA) (mean $\pm$ S.D., $n = 3$) in the lung tissue after a 180-min perfusion

The initial concentrations of NNK in the perfusate were 0.1 and 1.2 μ M. PHB-DNA adducts were not detected in any of the lung tissue samples.

DNA Adducts	NNK	NNK + PEITC	NNK	NNK + PEITC
	0.1 μ M	0.1 μ M	1.2 μ M	1.2 μ M
O^2 -POB-dThd	36.0 $\pm$ 2.9	9.9 $\pm$ 2.0 ^a	362.1 $\pm$ 11.1	89.2 $\pm$ 2.2 ^a
O^6 -POB-dGuo			43.7 $\pm$ 2.4	11.4 $\pm$ 2.1 ^a
7-POB-Gua			32.1 $\pm$ 3.2	18.0 $\pm$ 10.0
O^6 -Methyl-Gua			283.7 $\pm$ 14.0	162.8 $\pm$ 3.3 ^a

^a $p < 0.05$.

centration of keto alcohol (14.0 $\pm$ 0.4 pmol/ml) in the perfusate was greater than that of keto acid (8.0 $\pm$ 1.2 pmol/ml). NNAL (5.3 $\pm$ 0.4 pmol/ml) and its subsequent metabolites were formed to a lesser extent than the NNK metabolites. However, the subsequent metabolism of NNAL was analogous to that of NNK. NNAL-*N*-oxide (3.6 $\pm$ 0.4 pmol/ml) was the major NNAL metabolite, followed by diol (2.0 $\pm$ 0.2 pmol/ml). Hydroxy acid was not detected in the perfusate.

The metabolite profile in the tissue differed from that in the perfusate. In the tissue, hydroxy acid (0.77 $\pm$ 0.37 pmol/mg protein) and NNAL-*N*-oxide (0.32 $\pm$ 0.06 pmol/mg protein) were the major metabolites, accounting for approximately 42 and 17% of the total tissue metabolites (Fig. 3), respectively. The only DNA adduct that was detectable in the 0.1 μ M NNK perfusions was O^2 -POB-dThd (Table 2).

0.1 μ M NNK + 20 μ M PEITC. Figure 2, B, D, and F, illustrates the concentration-time data for NNK and its metabolites in the perfusate of the 0.1 μ M NNK + PEITC perfusions. Compared with the 0.1 μ M NNK perfusions, the metabolism of NNK was reduced in the presence of PEITC. Unmetabolized NNK (26.4 $\pm$ 3.0 pmol/ml) accounted for approximately 30 to 40% of the compounds at the end of the perfusion with PEITC compared with less than 5% when NNK was perfused alone. The observed decrease in NNK metabolism corresponded to changes in its apparent pharmacokinetic parameters (Table 1). There was a significant decrease in ER_{app} and CL_{app} from 0.13 $\pm$ 0.01 to 0.05 $\pm$ 0.01 and from 1.07 $\pm$ 0.09 to 0.39 $\pm$ 0.03 ml/min, respectively. Meanwhile, the $t_{1/2,app}$ increased significantly from 34 $\pm$ 5 to 108 $\pm$ 8 min in the presence of PEITC.

The formation of all the metabolites, except NNAL, was decreased with the addition of PEITC. NNK-*N*-oxide (17.0 $\pm$ 2.1 pmol/ml) remained the major metabolite in the perfusate, but only accounted for approximately 24% of the total perfusate metabolites, compared to the 49% that was observed when NNK was administered alone. Keto alcohol (9.5 $\pm$ 1.0 pmol/ml) still formed to a greater extent than keto acid (4.2 $\pm$ 0.2 pmol/ml), but the overall formation of both bioactivation metabolites was decreased. The concentration of NNAL (11.6 $\pm$ 1.1 pmol/ml) increased, whereas the formation of its subsequent metabolites, NNAL-*N*-oxide (2.0 $\pm$ 0.3 pmol/ml) and diol (1.2 $\pm$ 0.2 pmol/ml), decreased along with the other oxidative metabolites. To determine the effect of PEITC on metabolite formation, the AUC from time 0 to 180 min was calculated for each perfusate metabolite (Table 3). There was a significant decrease ($p < 0.05$) in AUC for all the oxidative metabolites and a significant increase in AUC for NNAL and NNK when PEITC was coadministered with NNK.

Although the concentration of metabolites in the perfusate changed significantly with the addition of PEITC, overall there was not a significant change in metabolite concentrations in the tissue. Hydroxy acid (0.70 $\pm$ 0.12 pmol/mg protein) remained the major tissue metabolite, followed by NNAL-*N*-oxide (0.35 $\pm$ 0.05 pmol/mg protein). Although tissue metabolite concentrations seemed unaffected, covalent binding in the tissue decreased significantly with the presence of PEITC. The level of O^2 -POB-dThd was significantly decreased from 36.0 $\pm$ 2.9 fmol/mg DNA when NNK was perfused alone to 9.9 $\pm$ 2.0 fmol/mg DNA when PEITC was coadministered (Table 2). The total covalent binding also decreased significantly from 0.25 $\pm$ 0.01 to 0.17 $\pm$ 0.02 pmol/mg protein.

1.2 μ M NNK. The perfusate concentration-time profiles of NNK and its metabolites in the 1.2 μ M NNK perfusions are depicted in Fig. 4, A, C, and E. Unmetabolized NNK (138 $\pm$ 45 pmol/ml) constituted approximately 16% of the compounds in the perfusate at the end of the perfusion, which was greater than the 5% in the 0.1 μ M NNK perfusions. The major perfusate metabolites were NNK-*N*-oxide (338 $\pm$ 64 pmol/ml) and keto alcohol (167 $\pm$ 29 pmol/ml). Overall, the metabolic profile in the perfusate was similar in the 1.2 and 0.1 μ M NNK treatments. However, the higher dose of NNK appeared to have an inhibitory effect on the overall metabolism, resulting in a CL_{app} of 0.66 $\pm$ 0.09 ml/min, an ER_{app} of 0.08 $\pm$ 0.01, and a $t_{1/2,app}$ of 64 $\pm$ 16 min (Table 1).

The major tissue metabolites were hydroxy acid (6.4 $\pm$ 1.9 pmol/mg protein) and NNAL-*N*-oxide (4.2 $\pm$ 1.2 pmol/mg protein), which accounted for approximately 32 and 21% of the total tissue metabolites (Fig. 5), respectively. With the higher dose of NNK, more individual DNA adducts were detected in the tissue (Table 2). O^2 -POB-dThd (362 $\pm$ 11 fmol/mg DNA) and O^6 -methyl-Gua (284 $\pm$ 14 fmol/mg DNA) were formed to a greater extent than O^6 -POB-dGuo (43.7 $\pm$ 2.4 fmol/mg DNA) and 7-POB-Gua (32.1 $\pm$ 3.2 fmol/mg

TABLE 3

The mean AUC_0^{180} (pmol · min/ml) $\pm$ S.D. of perfusate metabolites ($n = 3$)

	Keto Acid	NNAL- <i>N</i> -oxide	Diol	NNK- <i>N</i> -oxide	Keto Alcohol	NNAL	NNK
0.1 μ M	700 $\pm$ 120	270 $\pm$ 40	150 $\pm$ 20	4050 $\pm$ 200	1960 $\pm$ 90	720 $\pm$ 50	4550 $\pm$ 320
0.1 μ M + PEITC	370 $\pm$ 10 ^a	130 $\pm$ 20 ^a	70 $\pm$ 10 ^a	1550 $\pm$ 170 ^a	950 $\pm$ 80 ^a	1260 $\pm$ 80 ^a	8970 $\pm$ 350 ^a
1.2 μ M	6060 $\pm$ 840	2490 $\pm$ 750	1380 $\pm$ 410	36,700 $\pm$ 7400	19,400 $\pm$ 3500	10,700 $\pm$ 1300	78,000 $\pm$ 7100
1.2 μ M + PEITC	3400 $\pm$ 240 ^a	660 $\pm$ 320 ^a	450 $\pm$ 220 ^a	11,000 $\pm$ 1800 ^a	7700 $\pm$ 1500 ^a	12,900 $\pm$ 1100	96,700 $\pm$ 5000 ^a

^a $p < 0.05$ compared with the same concentration without PEITC.

FIG. 4. Concentration (picomoles per milliliter) $\pm$ S.D. versus time (minutes) profiles of NNK and metabolites in the perfusate of 1.2 μ M NNK (A, C, E) and 1.2 μ M NNK + PEITC (B, D, F) ($n = 3$). Symbol designations are as follows: NNK, $\bullet$; NNK-*N*-oxide, $\circ$; NNAL, $\blacksquare$; NNAL-*N*-oxide, $\square$; keto alcohol, $\blacktriangleleft$; keto acid, $\triangleleft$; diol, $\blacktriangle$. The profile of NNAL is replotted in E and F for comparison purposes. Where error bars are not apparent, they are smaller than the symbol.

DNA) in the tissue. None of the individual PHB-DNA adducts was present at detectable levels in the lung.

1.2 μ M NNK + 20 μ M PEITC. The presence of PEITC inhibited the metabolism of NNK (Fig. 4B). Approximately 48% of the final perfusate compounds was attributable to NNK (345 ± 34 pmol/ml), which was significantly greater than the 16% associated with NNK (138 ± 45 pmol/ml) when 1.2 μ M NNK was dosed alone. The formation of all the metabolites, except NNAL, was inhibited by PEITC (Fig. 4, D and F). The major perfusate metabolites were NNAL and NNK-*N*-oxide, which had comparable concentrations. The concentration of NNAL increased from 85.4 ± 8.1 pmol/ml when NNK was perfused alone to 124 ± 12 pmol/ml with the coadministration of PEITC. Meanwhile, the concentration of NNK-*N*-oxide decreased from 337 ± 64 to 120 ± 11 pmol/ml. The concentration of all the other oxidative metabolites decreased as well. The presence of PEITC was associated with a significant decrease in AUC from 0 to 180 min for all of the oxidative metabolites and a significant increase in the AUC of NNK (Table 3). A significant change in NNAL AUC was not observed. The addition of PEITC resulted in a decrease in CL_{app} from 0.66 ± 0.09 to 0.36 ± 0.03 ml/min. A decrease in ER_{app}

from 0.08 ± 0.01 to 0.04 ± 0.00 was also observed, whereas $t_{1/2,app}$ increased from 64 ± 16 to 149 ± 11 min (Table 1).

In the tissue, hydroxy acid (7.9 ± 1.9 pmol/mg protein) and NNK (2.5 ± 0.5 pmol/mg protein) had the highest concentrations. Overall there did not appear to be a consistent change in the tissue concentrations of metabolites with the addition of PEITC (Fig. 5). The reduction in NNK metabolism led to a decrease in covalent binding in the tissue. The estimated total covalent binding significantly decreased from 2.28 ± 0.02 to 1.38 ± 0.29 pmol/mg protein. The formation of individual DNA adducts also decreased significantly with the administration of PEITC (Table 2). The major DNA adduct in the tissue was *O*⁶-methyl-Gua (163 ± 3 fmol/mg DNA), followed by *O*²-POB-dThd (89.2 ± 2.2 fmol/mg DNA), 7-POB-Gua (18.0 ± 10.0 fmol/mg DNA), and *O*⁶-POB-dGuo (11.4 ± 2.1 fmol/mg DNA).

Discussion

Comparison to Previous IPRL Study. This study examined the effects of NNK concentration and the coadministration of PEITC on metabolism, metabolite distribution, and DNA adduct formation in the perfused rat lung. The final metabolite composition in the perfusate of

FIG. 5. Concentration of metabolites (picomoles per milligram of tissue) $\pm$ S.D. in the tissue after 180-min perfusion ($n = 3$). Open bars represent 1.2 μ M NNK, and shaded bars are 1.2 μ M NNK + PEITC. *, $p < 0.05$.

the 0.1 μ M NNK perfusions was similar to the 0.035 μ M NNK perfusion previously reported, despite a number of differences in the experimental designs (Schrader et al., 1998). Both studies found that a majority of the metabolites formed were from detoxification pathways (55%), whereas bioactivation pathways accounted for approximately 30% of metabolism. The extent of formation of most metabolites was in agreement between the two studies, with the exception of minor differences in the formation of diol and keto alcohol.

Whereas the extent of metabolite formation was comparable between the two studies, the estimated CL_{app} was lower in the present study (1.07 ± 0.9 ml/min) than in the previous study (2.1 ± 0.5 ml/min). The difference in CL_{app} may be attributable to the concentration difference of NNK between the two experiments because clearance was shown to decrease with increased concentration in the present study. Because the CL_{app} is much lower than perfusate flow, NNK is a low extraction ratio compound with respect to the lung.

The present study examined the metabolites in the lung tissue. Less than 4% of the radioactivity was recovered in the lung, and the metabolite profile of the tissue was different from that of the perfusate. Hydroxy acid was not detected in the perfusate in any of the treatment groups, but it was the major metabolite in the tissue for all the groups. This may be because of diffusional barriers within the lung. Hydroxy acid is the most hydrophilic of the metabolites and thus may not transverse the membranes as easily as the other more lipophilic metabolites, resulting in its accumulation in the lung tissue. In both studies the estimated apparent volumes of distribution were approximately equal to the volume of the reservoir, indicating that the partitioning of NNK and its metabolites into the lung tissue was not extensive.

It is important to note that the pharmacokinetic parameters that are reported here are apparent values. The equations that were used to estimate these parameters were derived based on the assumption that no reversible metabolism takes place in the system. Reconversion of NNAL to NNK is possible (Upadhyaya et al., 2000), but the extent of the reconversion in this system is unknown. If the reoxidation of NNAL to NNK is minor in the lung, then the estimates provided here should be fairly accurate. However, if the reconversion of NNAL to NNK is extensive, then the clearance of NNK from the lung reported here

would be underestimated. Studies investigating the reconversion of NNAL to NNK in the IPRL are currently in progress.

Effects of NNK Concentration. The dose-dependent carcinogenicity of NNK has been investigated in vivo (Hoffmann et al., 1984; Belinsky et al., 1990). In the study performed by Belinsky et al. (1990), doses of NNK ranging from 30 nmol (0.03 mg/kg) to 48,000 nmol (50 mg/kg) were administered to F344 rats subcutaneously three times a week for 20 weeks. The lowest dose administered (30 nmol/dose) induced lung tumors, but the formation was not significantly different from the control rats. The lowest dose that resulted in a significant formation of lung tumors was approximately 90 nmol/dose. The high dose used in the present study was the average of those two doses, 60 nmol, which achieved an initial perfusate concentration of 1.2 μ M. The 0.1 μ M perfusate concentration was the result of a 5-nmol NNK dose, which was the lowest achievable dose in initial perfusions when 50 μ Ci of [5- 3 H]NNK was administered. Given that both high- and low-dosing schemes had been used to test the tumorigenicity of NNK, it was of interest to determine the effects of dose on the metabolism and DNA adduct formation in the lung.

NNK at a concentration of 0.1 μ M was effectively metabolized in the lung. When the NNK concentration was increased 12-fold to 1.2 μ M, the efficiency of the lung metabolism appeared to decrease, as is evident by the decrease in apparent clearance. The increase in dose did result in a moderate decrease in the extent of formation of NNK-N-oxide, a modest increase in NNAL, and an increase in unmetabolized NNK at the end of the 180-min perfusion. NNK-N-oxide remained the major metabolite at 40% of the perfusate metabolites, as opposed to 50% at the lower dose. At the end of the 1.2 μ M perfusion, there was more unmetabolized NNK in the perfusate than after the 0.1 μ M perfusion. These results are supported by in vitro metabolism studies of NNK in alveolar type II cells. Those studies showed that higher doses of NNK reduce the formation of NNK-N-oxide and the other oxidative metabolites but increase the formation of NNAL and the level of unmetabolized NNK (Schrader et al., 2000). Another in vitro study by Richter et al. (2009) showed that NNK concentration did not greatly affect metabolite formation in the lung tissue of rats. However, they did observe that an increased NNK concentration was accompanied by an increase in NNAL at the "expense" of NNK-N-oxide. It is possible that higher doses of NNK saturated certain critical cytochrome P450 (P450) metabolic pathways, resulting in shunting through the NNAL pathway. However, the enzymes responsible for NNAL formation were apparently not efficient enough to compensate for the decrease in P450 activity, so overall less NNK was metabolized. The saturation of metabolism with increased NNK is also supported by the work of Devereux et al. (1988), which showed a decrease in the efficiency of *O*⁶-methyl-Gua formation with an increase in NNK.

*O*²-POB-dThd was the only DNA adduct detected in the tissue at the low concentration of NNK and was the major POB-DNA adduct detected in the lung of rats that were chronically treated with NNK in drinking water (Lao et al., 2006, 2007). No other DNA adducts were detected in the lung tissue of the 0.1 μ M perfusions. The metabolite profiles indicate that the intermediates for methyl and PHB-DNA adducts were formed, but those adducts were not detected. In the 1.2 μ M perfusions, all three POB-DNA adducts and *O*⁶-methyl-Gua were detected, but none of the PHB-DNA adducts were observed in the lung tissue. *O*²-POB-dThd remained the major POB-DNA adduct. There was slightly more *O*⁶-POB-dGuo than 7-POB-Gua in the lung, which was in contrast to the in vivo results that showed 7-POB-Gua at a significantly greater level than *O*⁶-POB-dGuo throughout the entire 20-week

bioassay (Lao et al., 2007). It is possible that there is an efficient DNA repair mechanism for O^6 -POB-dGuo that reduces its level in the rat lung in vivo but would not be present in the lung perfusion system. The formation of DNA adducts in the IPRL system further supports the idea that bioactivation in the lung, and not the liver, is responsible for the development of pulmonary tumors (Weng et al., 2007).

Effects of PEITC. PEITC inhibits the formation of NNK-induced lung tumors in rats (Morse et al., 1989; Hecht et al., 1996). The chemopreventive nature of PEITC has been attributed to its ability to inhibit P450 metabolism, resulting in a decrease in the bioactivation of NNK, thus decreasing the formation of potentially carcinogenic DNA adducts (Smith et al., 1990; Guo et al., 1991, 1992; von Weyarn et al., 2006). The addition of PEITC to the perfusion system resulted in a significant decrease in the formation of all the perfusate oxidative metabolites and an increase in NNAL formation, regardless of the NNK dose. The carbonyl reduction of NNK to NNAL is considered to be primarily catalyzed through non-P450 pathways, for example, by 11- β -hydroxysteroid, aldo-keto reductase, and carbonyl reductase (Atalla and Maser, 1999; Breyer-Pfaff et al., 2004), which could explain the apparent lack of inhibition of NNAL formation. In fact, the increase in NNAL formation could be because of shunting through the carbonyl reduction pathway as a result of the decrease in P450 oxidative metabolism. Previous in vitro studies also showed a decrease in the formation of oxidative metabolites with the treatment of PEITC (Smith et al., 1990; Guo et al., 1992; Staretz and Hecht, 1995; Staretz et al., 1997b). Whereas the in vitro studies did not show a significant increase in NNAL formation, in vivo studies showed a significant increase in NNAL and NNAL-glucuronide in the urine of rats that were coadministered PEITC with NNK, as well as a significant decrease in hemoglobin adducts resulting from α -hydroxylation (Hecht et al., 1996).

The decrease in oxidative metabolism consequently led to a decrease in DNA adduct formation and total covalent binding in the tissue as a result of α -hydroxylation. The addition of PEITC inhibited the formation of O^2 -POB-dThd and O^6 -POB-dGuo by 70 to 75% and decreased the formation of O^6 -methyl-Gua and 7-POB-Gua by 40 to 45%. These results are consistent with previous in vivo and in vitro studies that showed a decrease in DNA adduct formation with the addition of PEITC (Morse et al., 1989; Guo et al., 1991; Staretz et al., 1997a).

In summary, the current study shows that increased NNK concentration results in a significant decrease in the apparent pulmonary clearance, with only minor shifts in the metabolite profiles. The detection of DNA adducts indicate that metabolic activation in the lung leads to DNA damage and that bioactivation via the liver is not required. PEITC inhibits the formation of oxidative metabolites, which subsequently results in a decrease in DNA adduct formation. This study is the first to assess the pulmonary metabolism of NNK in the IPRL system that examined the time course of metabolite formation in the perfusate, quantitated the metabolites in the tissue, and measured the formation of individual DNA adducts. It is also the first study to examine the effect of PEITC on the pulmonary metabolism of NNK in the IPRL system. The results show the utility of the IPRL system in examining metabolism and DNA adduct formation in the lung.

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