CYP2A13: Variable Expression and Role in Human Lung Microsomal Metabolic Activation of the Tobacco-Specific Carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

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ABSTRACT

CYP2A13 is the most efficient cytochrome P450 enzyme in the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific lung carcinogen. The aims of this study were to determine the levels of CYP2A13 protein in human lung microsomes and to ascertain whether CYP2A13 plays any role in lung microsomal NNK metabolic activation. The expression of CYP2A6 and CYP2A13 was examined using a high-resolution immunoblotting method, following immunopurification with an anti-CYP2A5 antibody. We found that, of 116 human lung microsomal samples analyzed, 90% had detectable CYP2A6, whereas only 12% had detectable CYP2A13 with a detection limit of 2 fmol of CYP2A/mg protein. For the majority of microsomal samples analyzed, the level of CYP2A13 was found to be lower than the level of CYP2A6; overall, the highest level of CYP2A13 found (≈20 fmol/mg protein) was 10-fold lower than the highest level of CYP2A6 detected. Quantitative RNA-polymerase chain reaction analysis confirmed that the highly variable expression of the CYP2A proteins was consistent with variations in the levels of the corresponding CYP2A mRNAs in the same tissue samples. It is noteworthy that the level of CYP2A13, but not CYP2A6, was correlated with lung microsomal NNK metabolic activation activity. Furthermore, the addition of 8-methoxypsoralen, a CYP2A inhibitor, led to greater inhibition of NNK metabolic activation in microsomes containing relatively high levels of CYP2A13 than in samples containing no detectable CYP2A13. Taken together, these data indicate that human lung microsomal CYP2A13 is active in NNK metabolic activation. Therefore, individuals having relatively high levels of CYP2A13 expression will likely have an increased risk of developing smoking-related lung cancer.

The human cytochrome P450 (P450) CYP2A gene subfamily is known to have two functional members, CYP2A6 and CYP2A13 (Fernandez-Salguero et al., 1995; Su et al., 2000). Previous studies on CYP2A mRNA expression in human tissues indicated that CYP2A6 is expressed in the lung at much lower levels than in the liver, whereas CYP2A13 is selectively expressed in the respiratory tract (Koskela et al., 1999; Su et al., 2000). A more recent study indicated that CYP2A13 is also expressed in the bladder (Nakajima et al., 2006). Heterologously expressed CYP2A6 and CYP2A13 enzymes are both active in the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a lung procarcinogen, which is found in cigarettes (Hecht, 1998) and can also be produced endogenously from nicotine metabolites (Hecht et al., 2000). NNK metabolic activation involves P450-catalyzed hydroxylation at one of the two α carbons to the N-nitroso group, leading to the formation of reactive intermediates that can form either DNA adducts or else stable metabolites, including 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and 4-oxo-1-(3-pyridyl)-1-butanone (OPB) (Hecht, 1998). Although multiple human P450 enzymes, including CYP1A1, CYP1A2, CYP2A6, CYP2A13, CYP2B6, CYP2E1, and CYP3A4, can catalyze NNK α-hydroxylation, CYP2A13 is by far the most efficient, with $K_m$ values in the low micro-

ABBREVIATIONS: P450, cytochrome P450; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; OPB, 4-oxo-1-(3-pyridyl)-1-butanone; 8-MOP, 8-methoxypsoralen; HPLC, high-performance liquid chromatography; CHTN, Cooperative Human Tissue Network; PBS, phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, containing 136 mM NaCl and 2.7 mM KCl); BSA, bovine serum albumin; SF, Spodoptera frugiperda; PCR, polymerase chain reaction; RT, reverse transcription; NNAL, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol.
molar range (Su et al., 2000; Jalas et al., 2005). The catalytic efficiency of CYP2A13 is >100 times higher than that found for CYP2A6 (Jalas et al., 2005).

Several lines of evidence, including the high catalytic efficiency toward NNK, the tissue-selective expression of CYP2A13 mRNA in human respiratory tract, and the finding of a significant association between a reduced-activity CYP2A13 allele and decreased incidence of lung adenocarcinoma in light smokers (Wang et al., 2003), strongly suggest that CYP2A13 plays an important role in the metabolic activation of NNK in the respiratory tract of human smokers. The expression of CYP2A13 protein in a human tissue (nasal mucosa) was not demonstrated until recently, a success made possible by our development of a high-resolution immunoblotting method (Wong et al., 2005). There, we demonstrated that CYP2A13 protein is more abundant than CYP2A6 protein in human fetal nasal mucosa and that, whereas CYP2A6 is the major enzyme for coumarin hydroxylation, CYP2A13 is the main enzyme for NNK metabolic activation in human fetal nasal microsomes. More recently, CYP2A13 protein was found to be selectively expressed in airway epithelial cells in adult human lung, in an immunohistochemical study using a newly generated anti-CYP2A13 peptide antibody (Zhu et al., 2006). Nevertheless, it remained to be determined: 1) whether CYP2A13 protein can be detected in human lung microsomes, 2) whether microsomal levels of CYP2A13 protein differ among individuals, and 3) whether CYP2A13 plays a major role in NNK metabolism in lung microsomes. It should be noted that, although CYP2A6 protein was detected in human lung microsomes in earlier studies (Shimada et al., 1996; Bernauer et al., 2006), it could not be determined whether CYP2A6 or CYP2A13 was detected in those studies, given that the two proteins could not be distinguished under the conditions used.

Previous studies have demonstrated that human lung microsomes are active in the metabolic activation of NNK and that this microsomal reaction is at least partially catalyzed by P450 enzymes (Smith et al., 1992, 1995, 2003). A definitive identification of the specific P450 enzymes responsible for NNK metabolic activation in human lung microsomes was not possible in those studies, but the involvement of multiple P450s, including CYP2A6 and CYP2B6, was suggested. In the present study, we have estimated the expression levels of CYP2A6 and CYP2A13 proteins for a large number of adult human lung microsomal samples. We found that the levels of CYP2A6 and CYP2A13 proteins varied drastically among the samples analyzed. We subsequently performed quantitative analysis of CYP2A6 and CYP2A13 mRNA expression to establish whether the variable expression of the CYP2A proteins was accompanied by similar variations at the mRNA level. Furthermore, we then analyzed microsomal samples with varying levels of CYP2A protein expression for rates of NNK α-hydroxylation. In addition, a known CYP2A inhibitor, 8-methoxypsoralen (8-MOP), was used to confirm the participation of CYP2A6 or CYP2A13 in the microsomal metabolism of NNK. Our findings indicate that ~12% of lung microsomes studied had relatively high CYP2A13 protein expression and that these microsomes also had higher activities in NNK metabolic activation.

Materials and Methods

Chemicals and Enzymes. [5-3H]NNK (10.0–10.9 Ci/mmol; >98% pure) was purchased from Moravek Biochemicals (Brea, CA) and was further purified by reverse-phase high-performance liquid chromatography (HPLC) prior to use. NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and 8-MOP were purchased from Sigma-Aldrich (St. Louis, MO). Rat NADPH-cytochrome P450 reductase was obtained as described elsewhere (Zhang et al., 1998). Flo-Scint II scintillation fluid was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Microsomes containing heterogeneously expressed CYP2A6 (Liu et al., 1996) or CYP2A13 (Su et al., 2000) and polyclonal antibodies against mouse CYP2A5 (Gu et al., 1998) were prepared as described. All other chemicals and enzymes were purchased from Sigma-Aldrich or Bio-Rad (Hercules, CA).

Preparation of Human Lung Microsomes. The Institutional Review Board of New York State Department of Health approved the present study with human tissues. Adult human peripheral lung surgical resection specimens (noninvolved, adjacent tissue) were provided by the National Cancer Institute Cooperative Human Tissue Network (CHTN); tissues were obtained within 1 h after surgical removal and stored at ~80°C until use. All tissues were confirmed by CHTN to be histologically normal. The 116 tissue samples were obtained from 64 males and 52 females, ages 21 to 82 (~73% were between the ages of 51 and 70 years), comprising 93 Caucasians, 12 African Americans, and 11 of unknown ethnic origin. Clinical diagnosis for these patients included adenocarcinoma (42), squamous cell carcinoma (31), large-cell carcinoma (two), undetermined nonsmall cell carcinoma (five), other types of lung carcinoma (nine), secondary carcinoma (six), benign lung tumors (seven), nontumor diseases (six), or unknown/undetermined diseases (eight). Microsomes were prepared as described previously (Ding and Coon, 1990) and stored at ~80°C.

Immunopurification of CYP2A Proteins. Magnetic Dynabeads M-280 Tosylactivated (Invitrogen, Carlsbad, CA) were coated with rabbit anti-CYP2A5 IgG according to a protocol provided by the manufacturer. In brief, beads (2 × 109/ml) were washed once with 0.1 M borate buffer, pH 9.5, before they were incubated in the borate buffer with ~3 µg of IgG/106 magnetic beads for ~20 h at 37°C. The beads were then washed sequentially as follows: two washings in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) for 5 min at 4°C, one washing in blocking buffer (0.2 M Tris-chloride, pH 8.5, and 0.1% BSA) for 4 h at 37°C, one washing in PBS containing 0.1% BSA for 5 min at 4°C, one washing in 1% Tween 20 for 10 min at room temperature, and one washing in PBS containing 0.1% BSA for 5 min at 4°C. The final preparation of coated beads was stored at 4°C in PBS containing 0.1% BSA and 0.02% sodium azide at a concentration of ~2 × 109 beads/ml prior to use.

Human lung microsomal samples (0.5 mg of protein each) in 150 µl of microsome storage buffer (50 mM Tris-acetate, pH 7.4, 1.0 mM EDTA, and 20% glycerol) were mixed with 150 µl of a solubilization buffer [100 mM Tris-chloride, pH 7.4, 300 mM NaCl, 2.0% Triton X-100, 2.0% deoxycholate, 10 mM EDTA, and the Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. The mixtures were incubated, in a head-to-tail rotator, for 1 h at 4°C, followed by centrifugation at 4°C for 10 min, at 14,800g. The resultant supernatant fraction was removed and added to ~6.6 × 107 pelleted, antibody-coated beads, which were washed once with PBS containing 0.1% BSA before use. The coated beads were incubated with the solubilized microsomal proteins overnight at 4°C in a head-to-tail rotator. The beads were subsequently washed, at 4°C, once in PBS containing 0.05% Tween 20 and once in PBS alone, and the captured proteins were eluted by mixing the pelleted beads with 16 µl of the Laemmli sample buffer (Bio-Rad), followed by centrifugation at 4°C for 5 min at 14,800g. The beads were applied to a magnetic concentrator (Invitrogen), and the immunopurified proteins were stored at ~20°C before immunoblot analysis.
Immunoblot Analysis. High-resolution SDS-polyacrylamide gel electrophoresis was performed using a DNA sequencing apparatus (Bio-Rad) as described previously (Wong et al., 2005), with minor modifications. The immunopurification products (16 μl), mixed with 0.5 μl of β-mercaptoethanol and 20 μg of Sf9 insect cell microsomal protein (as carrier protein), were boiled for 5 min before loading onto the gel. All samples were analyzed on 10% SDS-polyacrylamide (37.5:1, acrylamide/bis-acrylamide) gels (0.75 mm thick), with the use of standard Laemmli buffers (Laemmli, 1970). Electrophoresis was carried out for 24 h, at room temperature, in the constant-current mode, at 15 mA for stacking and 11 to 18 mA for separation. A cooling fan was used, and the electrophoresis buffer was replenished at least three times during the separation. After electrophoresis, proteins were transferred to a nitrocellulose membrane (0.45 μm; Bio-Rad).

Immunoblot analysis was performed essentially as described previously (Ding and Coon, 1990), with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and a rabbit anti-CYP2A5 antibody (Gu et al., 1998). Heterologously expressed CYP2A6 and CYP2A13, contained in Sf9 microsomes, were used as positive controls. The nitrocellulose membranes were incubated with the CYP2A5 antisera (1:2000) overnight at 4°C and then for an additional 60 min at room temperature. Densitometric analysis of scanned gel images was carried out with the use of LabWorks, version 4.5 (UVP, Upland, CA). Estimates of the amounts of CYP2A6 and CYP2A13 proteins were obtained by comparing the signal intensity of a given sample to the intensities of CYP2A6 and CYP2A13 protein standards that were analyzed on the same gel.

Quantitative Analysis of CYP2A6 and CYP2A13 mRNA Expression. Total RNA was isolated from lung tissues with the use of the RNaseasy Plus Mini kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 1.0 μg of total RNA in a total volume of 20 μl, using the SuperScript III first-strand synthesis system and an Oligo(dT)20 primer (Invitrogen). Real-time PCR was carried out using the supermix 5700 Fast real-time PCR system and the SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA). The mRNA levels were determined for CYP2A6, CYP2A13, and β-actin. The primers used for CYP2A6 and CYP2A13 proteins were obtained by comparing the signal intensity of a given sample to the intensities of CYP2A6 and CYP2A13 mRNA standards that were analyzed on the same gel. The PCR was carried out in a reaction volume of 20 μl. Reaction mixtures for quantification of CYP2A6 or CYP2A13 mRNA contained 5 μl of 10-fold-diluted RT product, 2.0 μl of 10× SYBR Green PCR buffer, 1.6 μl of dNTP mix (containing 2.5 mM each of dATP, dCTP, and dGTP and 5.0 mM dUTP), 3.0 mM MgCl₂, 0.25 μM (for CYP2A6) or 0.3 μM (for CYP2A13) of each primer, 0.2 units of AmpliTaq Gold DNA polymerase. The reaction mixture for β-actin mRNA quantification was the same as that for CYP2A13, except that 2 μl of 10-fold-diluted RT product and 2.5 mM MgCl₂ was used, and the AmpliTaq Gold enzyme was omitted. Reactions for CYP2A6 and CYP2A13 were initiated by incubation at 50°C for 2 min (to allow degradation of any contaminating PCR products by the AmpliTaq Gold) and then denaturation at 95°C for 10 min (to inactivate the AmpliTaq Gold), followed by 50 cycles of amplification (95°C for 15 s, 64°C for 15 s, and 72°C for 30 s). For β-actin, the initial incubation at 50°C was omitted, and the PCR was performed for 40 cycles (95°C for 15 s, 63°C for 10 s, and 72°C for 30 s). Data collection was performed at 72°C. All reactions were performed in duplicate. Each PCR run included reactions using standard templates (10-fold serial dilutions of cloned cDNA for CYP2A6 and CYP2A13 or 10-fold serial dilutions of gel-purified RT-PCR product of β-actin) and a no-template control, in addition to reactions using the standard lung cDNA. Standard curves were generated by plotting the values of the threshold-crossing points against log-transformed copy numbers of standard templates. The levels of CYP2A6 and CYP2A13 mRNAs in various total RNA preparations were normalized by the level of β-actin mRNA in a given sample.

Detection of the Occurrence of Variant CYP2A Genes and Transcripts. Sequence analysis of the CYP2A13 exons and exon-intron junctions was performed as described previously (Zhang et al., 2002, 2003) to detect any occurrence of genetic polymorphisms. Potential variations in CYP2A13 mRNA sequence, resulting from alternative splicing, were detected via RNA-PCR; the CYP2A13 mRNA was amplified in four fragments, spanning exons 1 to 3, 3 to 6, 6 to 7, and 7 to 9, with the use of primers 5'-tgtgttgtgtttgctggc-3' and 5'-ccgaggggactccgttctctg-3' and 5'-gcgggacatctcactcag-3' and 5'-aaggagctacggcgcgaa-3'. The sizes of the PCR products were compared with those amplified from lung samples that did not contain the CYP2A-X protein. To detect potential occurrence of mRNAs corresponding to CYP2A7 or CYP2A6/CYP2A7 hybrid (CYP2A6*3), RNA-PCR analysis was performed using PCR primers complementary to CYP2A6 and CYP2A7 exons 7 (5'-ggccgagatctcactcag-3') and 9 (5'-aagtctctagttgctaggg-3').

Assay for NNK Metabolism. The contents of the reaction mixtures are described in the notes to tables. The reaction mixtures were preincubated for 1 min at 37°C, prior to the addition of NADPH. Reactions were terminated by the addition of 25 μl each of 25% zinc sulfate and saturated barium hydroxide. The samples were then spun, and 50 μl of the supernatant fraction was analyzed on a reverse-phase HPLC system equipped with a Radiomatic series A-500 on-line radioactivity detector (Perkin-Elmer) and a Waters μBondapak C18 column (3.9 × 300 mm; 10 μm; Waters, Milford, MA). The analytes were eluted, following an initial wash with 100% A (20 mM Tris-chloride, pH 6.0) for 1 min, by a linear gradient from 100% A to 70% A and 30% B (95% methanol) over 74 min, at a flow rate of 1 ml/min. Sodium bisulfite, a trapping agent for OPB, was found to interfere with detection of radiolabeled OPB under conditions that permit separation of 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and HPB, as described previously by others (Smith et al., 1995). Therefore, sodium bisulfite was not included in the reaction mixture or the mobile phase. Under these conditions, OPB and HPB were together detected as a single peak (Smith et al., 1995), which represented abundance of total metabolites formed through the two differing α-hydroxylation pathways (Peterson et al., 1991).

Other Methods. Statistical significance of differences between two groups was analyzed using Student's t test or Mann-Whitney rank sum test (for data that failed the normality test or equal-variance test), and statistical significance of differences among multiple groups was analyzed using one-way analysis of variance. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Results

Detection of CYP2A6 and CYP2A13 Proteins in Adult Human Lung Microsomes. Expression of CYP2A6 and CYP2A13 proteins was investigated by immunoblot analysis with the use of anti-CYP2A5 antibody. This antibody is known to cross-react with both human CYP2A6 and CYP2A13 but not with 12 other human P450 enzymes examined on immunoblots, including CYP1A1, CYP1A2, CYP2B6, CYP2E1, CYP3A4, CYP1B1, CYP2C8, CYP2C9-Arg, CYP2C9, CYP2D6-Val, CYP3A5, and CYP4A11 (Zhuo et al., 1999; Su et al., 2000). The CYP2A6 and CYP2A13 proteins, which could not be resolved on a conventional SDS-polyacrylamide gel (Gu et al., 2000), were separated using a 40-cm-long gel; the electrophoresis was carried out for 24 h in a DNA sequencing apparatus. The sensitivity of CYP2A detection was increased by immunopurification of...
CYP2A proteins using an anti-CYP2A5 antibody prior to electrophoresis. CYP2A6 and CYP2A13 proteins were identified on the basis of migration with recombinant CYP2A proteins, whereas the levels of the CYP2A proteins were estimated through comparisons with known amounts of CYP2A protein standards.

CYP2A6 and CYP2A13 were both detected in adult human lung microsomes (Fig. 1, A and B). A CYP2A-immunoreactive unknown protein (designated CYP2A-X), which migrated more slowly than did either CYP2A6 or CYP2A13, was also detected in a small number of samples (Fig. 1C). The results from all immunoblot experiments are summarized in Table 1. Of the 116 human adult lung microsomal samples analyzed, CYP2A6 was detected in 104 (89.7%) samples (87 with CYP2A6 alone, 13 with both CYP2A6 and CYP2A13, and four with CYP2A6 and CYP2A-X), whereas CYP2A13 was detected in 14 (12.1%) samples (one with CYP2A13 alone and 13 with both CYP2A13 and CYP2A6). CYP2A-X was detected in 5 of the 116 (4.3%) samples (one with the unknown protein alone and four with both CYP2A-X and CYP2A6). No CYP2A-immunoreactive protein was detected in 10 of the 116 (8.6%) samples. Lane 1, 20 fmol of recombinant CYP2A13 (no immunopurification). B, representative samples with high or low CYP2A6 expression but no detectable CYP2A13 expression. Lanes 1 to 5, immunopurified CYP2A from various lung microsomal samples; lane 6, immunopurified CYP2A from a mixture of recombinant CYP2A6 and CYP2A13, 25 fmol each; lane 7, 20 fmol of recombinant CYP2A13 (no immunopurification). B, representative samples with positive CYP2A13 expression and varying levels of CYP2A6 expression. Lanes 1 to 6, immunopurified CYP2A from various lung microsomal samples; lane 7, immunopurified CYP2A from a mixture of recombinant CYP2A6 and CYP2A13, 25 fmol each. The images were compiled from five separate gels. C, example of the unknown, CYP2A-immunoreactive protein (CYP2A-X), detected in selected lung microsomal samples. Lanes 1, 20 fmol of recombinant CYP2A13 (no immunopurification); lanes 2 and 3, immunopurified CYP2A from a mixture of recombinant CYP2A6 and CYP2A13, 25 fmol each. The images were compiled from five separate gels. C, example of the unknown, CYP2A-immunoreactive protein (CYP2A-X), detected in selected lung microsomal samples. Lanes 1, 2, and 5, 50 µg of protein/lane) but not others (lanes 3 and 4; 50 µg of protein/lane). Lanes 6 and 7, recombinant CYP2A6 and CYP2A13, respectively; 10 fmol each. +, lanes containing recombinant CYP2A protein standards.

Remarkable interindividual variations were found for levels of the CYP2A proteins detected in human lung microsomes (Fig. 1). Recovery of recombinant CYP2A6 and CYP2A13 in the immunopurification step was estimated to be ~70 to 80% for both proteins (data not shown). By assuming that the recovery of CYP2A proteins from various lung microsomal preparations was constant, we estimate that the amounts of CYP2A6 protein detected in human lung microsomes ranged from ≤2 (detection limit) to ~220 fmol/mg microsomal protein and that the amounts of CYP2A13 protein ranged from ≤2 (detection limit) to ~20 fmol/mg microsomal proteins. The detection limit was estimated by immunoblot analysis of varying amounts of recombinant CYP2A proteins.

In experiments not shown, we attempted to identify the CYP2A-X protein. We hypothesized that CYP2A-X corresponds to a splice variant of CYP2A13 or, less likely, CYP2A6, given the cross-reactivity of CYP2A-X with the anti-CYP2A antibody and the size difference between CYP2A-X and either CYP2A6 or CYP2A13; the variant is more likely related to CYP2A13 than it is related to CYP2A6, given that CYP2A13, but not CYP2A6, was never detected in CYP2A6-containing samples (Table 1). Alternatively, we reasoned that CYP2A-X might correspond to CYP2A7 or CYP2A6*3, the gene conversion product of CYP2A6 and CYP2A7 (Fernandez-Salgueiro et al., 1995); neither the CYP2A7 nor CYP2A6*3 protein had been detected in human lung before. We found, through sequence analysis of the CYP2A13 exons and exon-intron junctions for one of the CYP2A-X-containing tissue samples, that the DNA sample had only sequences corresponding to the CYP2A13*1 allele; therefore, CYP2A-X is unlikely to be a CYP2A13 allelic variant. An analysis of the sizes of CYP2A13 RNA-PCR products from three CYP2A-X-containing samples did not reveal any CYP2A13 splicing variants. Further RNA-PCR analysis of the three samples detected cDNA sequence corresponding to exons 8 of CYP2A6, but not that of CYP2A7, a finding indicative of the absence of either CYP2A7 or the CYP2A6/CYP2A7 hybrid (CYP2A6*3) mRNA. Thus, although the identity of CYP2A-X remains to be determined, this unknown, CYP2A-related protein appears not to be encoded by CYP2A7 or CYP2A6*3, nor does it correspond to a CYP2A13 splicing variant.

Correlation of CYP2A mRNA Expression with CYP2A Protein Expression in Human Lung Tissues. To determine whether the interindividual differences in the expression of CYP2A proteins were concordant with interindividual differences in CYP2A mRNA levels in adult lung tissues, we selected tissue samples having high or low CYP2A protein levels for CYP2A mRNA quantification. For the analysis of CYP2A6 expression, we selected seven samples with relatively high levels of microsomal CYP2A6 protein (CYP2A6-High) and nine samples with low or undetectable levels of CYP2A6 protein (CYP2A6-Low). We found (Table 2) that the abundance of CYP2A6 mRNA, normalized by the abundance of β-actin mRNA, was significantly higher in the CYP2A6-High group than in the CYP2A6-Low group (84 ± 71 versus 24 ± 29 copies/10⁶ copies of β-actin, mean ± S.D.; P < 0.05), a result indicative of a positive correlation between levels of CYP2A6 protein and CYP2A6 mRNA in the lung.

For the analysis of CYP2A13 expression, we selected eight samples with detectable levels of CYP2A13 protein (CYP2A13-High) and 16 samples with no detectable CYP2A13 protein (CYP2A13-Low). We found (Table 2) that samples of the CYP2A13-High group contained higher levels of CYP2A13...
mRNA than did samples of the CYP2A13-Low group (median, 3.0 (0.3, 14) copies of CYP2A13/106 copies of 2-tubulin mRNA) than did samples of the CYP2A13-High group (median, 15 (5, 34) copies of CYP2A13/106 copies of 2-tubulin mRNA). Comparisons between CYP2A13-High and CYP2A13-Low groups failed equal variance and normality tests. Significantly lower than CYP2A13-High; $P < 0.05$, Student’s $t$ test. Significantly lower than CYP2A6-High; $P < 0.05$, Student’s $t$ test. Comparisons between CYP2A13-High and CYP2A13-Low groups failed equal variance and normality tests. Significantly lower than CYP2A6-High; $P < 0.05$, Mann-Whitney rank sum test.

TABLE 2

<table>
<thead>
<tr>
<th>Tissue Samples</th>
<th>n</th>
<th>CYP2A6</th>
<th>CYP2A13</th>
<th>CYP2A-X</th>
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<tr>
<td></td>
<td></td>
<td>% of Total</td>
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<td></td>
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<tr>
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<tr>
<td>CYP2A6-Low</td>
<td>16</td>
<td>ND</td>
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</tbody>
</table>

$^a$ Below detection limit (2 fmol/mg microsomal protein).

$^b$ Significantly lower than CYP2A6-High; $P < 0.05$, Student’s $t$ test.

$^c$ Comparisons between CYP2A13-High and CYP2A13-Low groups failed equal variance and normality tests.

$^d$ Significantly lower than CYP2A6-High; $P < 0.05$, Mann-Whitney rank sum test.

Human lung microsomes were grouped into three categories for determination of in vitro NNK metabolism: samples containing no detectable CYP2A13 protein but either high (group 1) or low (group 2) levels of CYP2A6 protein and samples containing detectable levels of CYP2A13 protein and varying levels of CYP2A6 (group 3). As shown in Table 3, group 1 and group 2 microsomal samples did not differ significantly in their rates of NADPH-dependent NNK $\alpha$-hydroxylation (0.61 versus 0.64 pmol/min/mg protein; $P > 0.05$), indicating that, with NNK at 10 $\mu$M, CYP2A6 did not contribute substantially to NNK metabolic activation in human lung microsomal reactions. In contrast, group 3 microsomes had significantly higher rates of NNK $\alpha$-hydroxylation than did microsomes in groups 1 and 2 (combined) (0.87 versus 0.62 pmol/min/mg protein), suggesting that CYP2A13 protein does contribute to NNK metabolic activation in human lung microsomes that have relatively high CYP2A13 expression. On the other hand, the three groups did not differ significantly in their rates of NNAL formation (Table 3), a finding consistent with the lack of involvement of either CYP2A6 in the carbonyl reduction of NNK. In experiments not presented, coumarin 7-hydroxylase activity (a representative activity for CYP2A6) was detected in group 1 microsomes but not in group 2 microsomes. This observation supports the notion that CYP2A6 protein is functional in human lung
protein levels showed not only higher rates of total HPB/OPB formation but also greater extent of inhibition (46%) by 8-MOP than did microsomes with low CYP2A13 protein expression (31% inhibition). A relatively low concentration of 8-MOP was chosen so as to minimize inhibition of P450 enzymes other than CYP2A6 or CYP2A13 (Koenigs et al., 1997; Zhang et al., 2001); the amount used (2.5 μM) was found to be sufficient to inhibit >90% of the activity of recombinant CYP2A13 toward 10 μM NNK (data not shown). In contrast, the NNK α-hydroxylation activity of CYP2A13-Low microsomes with either high or low CYP2A6 protein showed not only similar rates of total HPB/OPB formation but also a similar extent of inhibition by 8-MOP (data not shown), consistent with the lack of a significant role for CYP2A6 in this reaction. As an internal control, NADPH-dependent formation of NNAL was not affected by the addition of 8-MOP (Table 4). In other studies not presented, the addition of IgG to reaction mixtures increased the rates of NADPH-dependent product formation, a phenomenon that made it impossible to determine the specific effects of anti-2A antibody on NNK metabolism in these lung microsomes.

**Fig. 2.** HPLC analysis of NNK metabolites formed by human lung microsomes. Assays for NNK metabolism were performed as described under Materials and Methods, with the use of 10 μM [5-3H]NNK. Contents of incubation mixtures are described in the notes to Table 3. Metabolites were analyzed using an HPLC system equipped with an on-line radioactivity detector. Representative chromatograms for three different types of incubation mixtures are shown. A, complete reaction mixtures that contained boiled human lung microsome. B, no-NADPH control reactions. C, complete reaction mixtures, with lung microsomes that contained CYP2A13. The same microsomal preparation was used for the reactions shown in B and C.

**TABLE 3**

NADPH-dependent NNK α-hydroxylation and NNAL formation by human lung microsomes with varying expression levels of CYP2A6 and CYP2A13 proteins

<table>
<thead>
<tr>
<th>Group</th>
<th>Rates of Metabolite Formation</th>
<th>HPB and OPB a</th>
<th>NNAL b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg microsomal protein</td>
<td>pmol/min/mg microsomal protein</td>
<td>pmoles/min/mg microsomal protein</td>
</tr>
<tr>
<td>1, CYP2A6-High/CYP2A13-Low (n = 10)</td>
<td>0.61 ± 0.29</td>
<td>49 ± 16</td>
<td></td>
</tr>
<tr>
<td>2, CYP2A6-Low/CYP2A13-Low (n = 12)</td>
<td>0.64 ± 0.20 b</td>
<td>41 ± 7 b</td>
<td></td>
</tr>
<tr>
<td>3, CYP2A6-High or Low/CYP2A13-High (n = 10)</td>
<td>0.87 ± 0.15 c</td>
<td>49 ± 21 c</td>
<td></td>
</tr>
</tbody>
</table>

a The two products of NNK α-hydroxylation were not resolved under the HPLC conditions used.

b Not significantly different from groups 1; *P > 0.05*, Student’s *t* test.

c Groups 1 and 2, both having no detectable CYP2A13 protein, are combined, for comparisons with group 3, which has detectable CYP2A13.

d Significantly different from groups 1 and 2; *P < 0.05*, Student’s *t* test.

e Not significantly different from groups 1 and 2; *P > 0.05*, Student’s *t* test.

**Discussion**

Critical to the hypothesis that CYP2A13 plays an important role in tobacco-related human lung tumorigenesis is the assumption that CYP2A13 protein is expressed in human lung, where it is active in the metabolic activation of NNK. However, although CYP2A13 protein has been detected in human fetal nasal mucosa through the use of a high-resolution immunoblot method (Wong et al., 2005) and in human lung through immunohistochemical analysis of tissue sections using an anti-CYP2A13 peptide antibody (Zhu et al., 2006), a quantitative assessment of both the levels of CYP2A13 protein expression and the extent of the enzyme’s interindividual variations was not possible until now.

In the present study, we have, for the first time, successfully detected CYP2A13 protein in human lung microsomal preparations. Our ability to resolve and detect CYP2A6 and CYP2A13 proteins in human lung microsomes, through the use of our recently developed high-resolution immunoblot method (Wong et al., 2005), combined with the newly adopted use of our recently developed high-resolution immunoblot method (Wong et al., 2005), combined with the newly adopted
TABLE 4

Inhibition of human lung microsomal NNK α-hydroxylation by 8-methoxypsoralen

NNK metabolism assays were performed as described in the notes to Table 3, except that 8-methoxypsoralen (dissolved in methanol) was added to the reaction mixture at a final concentration of 2.5 μM. At the amount used (1%, final concentration), methanol had no effect on the rate of NNK metabolism. Microsomes containing 5 to 20 (CYP2A13-High) or <2 (CYP2A13-Low) fmol/mg CYP2A13 protein were analyzed. Values presented are means ± S.D. CYP2A-dependent rate, difference between rate determined without 8-MOP addition and rate determined with 8-MOP addition. There was no significant difference among the four rates of NNAL formation (one-way analysis of variance, P > 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Rates of Metabolite Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>HPB and OPB</td>
</tr>
<tr>
<td>CYP2A13-High</td>
<td>4</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>CYP2A13-Low</td>
<td>4</td>
<td>0.51 ± 0.20</td>
</tr>
<tr>
<td>CYP2A13-High</td>
<td>5</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td>CYP2A13-Low</td>
<td>5</td>
<td>0.34 ± 0.08</td>
</tr>
</tbody>
</table>

* CYP2A13-High vs. CYP2A13-Low, in the absence of 8-MOP, P < 0.05, Student’s t test.
* CYP2A13-High vs. CYP2A13-Low, in the presence of 8-MOP, P > 0.05, Student’s t test.
* CYP2A13-High vs. CYP2A13-Low, P < 0.05, Mann-Whitney rank sum test.

Inhibitory activity was determined using either HPB or OPB CYP2A13. The S.D. values are given in Table 4. A Student’s t test was used to compare the CYP2A13 activity levels in the presence and absence of 8-MOP. The HPB CYP2A13 was used to determine the CYP2A13 activity levels in the presence of 8-MOP. A Student’s t test was used to compare the CYP2A13 activity levels in the presence and absence of 8-MOP.

The level of CYP2A13 protein detected here in human lung microsomes resulted in the formation of products from both α-hydroxylation and carbonyl reduction, as previously reported (Smith et al., 1992, 1995, 2003), with NNAL being the major product when the substrate is at micromolar concentrations. It was striking that much higher rates of NNK α-hydroxylation were found in our study than those reported in three previous studies (Smith et al., 1992, 1995, 2003). One difference between our study and those reported previously was that the time between tissue excision and tissue freezing was 1 h or less in the present study, whereas in the others, it was reportedly 6 to 11 h (Smith et al., 1992), 0.25 to 5 h (Smith et al., 1995), or not specified (Smith et al., 2003).

The level of CYP2A13 protein detected here in human lung microsomes was much lower than that found previously in human fetal nasal mucosal microsomes (~1.6 pmol/mg protein); in the latter, CYP2A13 protein was readily detected, without the need for immunopurification (Wong et al., 2005). The lower expression of CYP2A13 protein in human lung

use of immunopurification for enrichment of CYP2A proteins prior to immunoblot analysis, allowed us to estimate the expression levels for the two highly similar P450 proteins in 116 human lung microsomal samples. Our results demonstrate that both CYP2A6 and CYP2A13 proteins are expressed in human lung, but their expression levels vary dramatically among the samples analyzed. We further confirmed that, overall, the expression of CYP2A6 and CYP2A13 proteins correlated with the expression of the corresponding mRNAs in the same tissue samples.

CYP2A6 and CYP2A13 proteins were detected in ~90 and ~12%, respectively, of lung microsome samples examined. The notion that those samples that failed to show detectable CYP2A proteins do nevertheless contain the CYP2A proteins, albeit at levels below our detection limit (~2 fmol/mg microsomal protein), is supported by: 1) the positive detection of corresponding CYP2A mRNAs in most of the specimens analyzed, 2) the greater sensitivity of PCR-based mRNA detection over the sensitivity of protein detection on immunoblot, and 3) the recent report of localized expression of CYP2A13 protein in airway epithelial cells (Zhu et al., 2006).

For the majority of microsomal samples analyzed, the level of CYP2A13 was found to be lower than the level of CYP2A6. The content of CYP2A13 protein in the lung microsomal preparations examined was estimated to be not higher than 20 fmol/mg protein, a level ~10-fold lower than the highest level of CYP2A6 protein detected. It is noteworthy that we had previously found (in a much smaller set of human lung samples) that CYP2A13 mRNA was present at higher levels than CYP2A6 mRNA (Su et al., 2000). Given the large interindividual variations in the expression levels of the two CYP2A genes, it is possible that the number of samples analyzed in the previous study was too small to be representative of the general population. Remarkably, we found in the present study that the level of CYP2A13 protein, but not CYP2A6 protein, was correlated with lung microsomal NNK metabolic activation activity. Furthermore, the addition of 8-MOP, a potent CYP2A inhibitor, led to greater inhibition of NNK metabolic activation in CYP2A13-High microsomes than in CYP2A13-Low samples. Taken together, these data indicate that human lung microsomal CYP2A13 is active in NNK metabolic activation; accordingly, individuals who have relatively high levels of pulmonary CYP2A13 expression will probably have an increased risk of developing smoking-related lung cancer. Likewise, for lung toxicants that are preferentially activated by CYP2A6, individuals with high CYP2A6 expression in the lung will probably have greater risk of chemical-induced lung toxicity than will those individuals with little CYP2A6 expression.

The observed large variation in pulmonary CYP2A6 and CYP2A13 expression is unlikely to be due to variations in the quality of tissue specimens used, given that many samples having no detectable CYP2A13 did have relatively high CYP2A6 levels, whereas samples with a level of CYP2A13 higher than the level of CYP2A6 were also observed. On the other hand, given the reported higher expression levels of CYP2A13 protein in lung airway epithelial cells than in other lung cells (Zhu et al., 2006), the extent of variation in CYP2A13 expression might have been confounded to some extent by variations in the abundance of epithelial cells in each biopsy samples. However, the extent of variation in cellular composition among the biopsy samples studied is expected to be small, given that all tissue samples are from peripheral lung and that we did not notice any obvious, large airway structures during tissue processing. The genetic, epigenetic, and environmental factors that influence CYP2A expression in the lung are subjects of ongoing studies in the Ding laboratory. In this regard, we have identified a CYP2A13 7520C>G variation that is associated with decreased CYP2A13 expression in human lung (Zhang et al., 2004), and we have also obtained evidence supporting the involvement of epigenetic factors in CYP2A13 regulation (Ling et al., 2007).

Metabolism of NNK by human lung microsomes resulted in the formation of products from both α-hydroxylation and carbonyl reduction, as previously reported (Smith et al., 1992, 1995, 2003), with NNAL being the major product when the substrate is at micromolar concentrations. It was striking that much higher rates of NNK α-hydroxylation were found in our study than those reported in three previous studies (Smith et al., 1992, 1995, 2003). One difference between our study and those reported previously was that the time between tissue excision and tissue freezing was 1 h or less in the present study, whereas in the others, it was reportedly 6 to 11 h (Smith et al., 1992), 0.25 to 5 h (Smith et al., 1995), or not specified (Smith et al., 2003).

The level of CYP2A13 protein detected here in human lung microsomes was much lower than that found previously in human fetal nasal mucosal microsomes (~1.6 pmol/mg protein); in the latter, CYP2A13 protein was readily detected, without the need for immunopurification (Wong et al., 2005). The lower expression of CYP2A13 protein in human lung
than in human nasal mucosa is consistent with previous findings for CYP2A13 mRNA expression (Su et al., 2000). However, given that CYP2A13 is preferentially expressed in airway epithelial cells (Zhu et al., 2006), the concentration of CYP2A13 in these cells would be much higher than was detected in whole-lung microsomes, in which the cells containing relatively high levels of CYP2A13 protein are mixed with a much larger pool of cells with little CYP2A13 expression. By the same token, the rates of NNK α-hydroxylation were, on average, only ~40% higher in microsomes containing 3 to 20 fmol/mg CYP2A13 than in microsomes containing <2 fmol/mg CYP2A13; the rate difference would be much greater if the rates were normalized by total protein in airway epithelial cells, rather than by proteins of all lung cells. In support of this idea, a previous study had reported increased formation of NNK α-hydroxylation products in an enriched human alveolar type II cell preparation, compared with the amounts formed by a mixed population of lung cells or an enriched alveolar macrophage preparation (Smith et al., 1999). Even in individuals with <2 fmol CYP2A13/mg microsomal protein, CYP2A13 may still play an important role in NNK metabolic activation in airway epithelial cells, a notion supported by the 30% reduction in activity rendered by the addition of 8-MOP to CYP2A13-Low samples.

The enzymes responsible for the remaining microsomal activities seen in the presence of 8-MOP have yet to be identified. In previous studies, several P450 enzymes were found, on the basis of inhibition by antibodies or chemical inhibitors, to contribute to human lung microsomal NNK α-hydroxylation (Smith et al., 1995, 2003); these included CYP2A, CYP2B6, CYP2E1, and CYP3A4/5, of which only CYP2B6 has a $K_m$ value for NNK that is close to that of CYP2A13 (Jalas et al., 2005). Although the catalytic efficiency of CYP2B6 toward NNK is much lower than that of CYP2A13, CYP2B6 seemed to be more efficient than CYP2A6 or the other human P450 enzymes that are known to be active toward NNK (Jalas et al., 2005). It is noteworthy that the finding that addition of antibody preparations (e.g., anti-CYP2A6 or control IgG) to incubation mixtures can lead to increased NNK metabolism (Smith et al., 1995, 2003), an observation confirmed in the present study (data not shown), suggests that the antibody inhibition approach is not efficient for lung microsomal preparations with very low activity toward NNK α-hydroxylation. A similar lack of antibody inhibition of CYP2A activity was reported for human bladder cells. In support of this idea, a previous study had reported increased NNK metabolism (Smith et al., 1995, 2003), an observation confirmed in the present study (data not shown), which suggests that the antibody inhibition approach is not efficient for lung microsomal preparations with very low activity toward NNK α-hydroxylation. A similar lack of antibody inhibition of CYP2A activity was reported for human bladder cells.

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