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Baculovirus-Mediated Expression and Characterization of Rat CYP2A3 and Human CYP2A6: Role in Metabolic Activation of Nasal Toxicants

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SUMMARY

Cytochrome P450 2A3 (CYP2A3) was previously identified in rat lung by cDNA cloning and recently found to be expressed at a high level in the olfactory mucosa. In the current study, CYP2A3 was expressed in insect cells lacking endogenous cytochrome P450 (P450) activity, and the substrate specificity of the recombinant cytochrome was characterized and compared with that of CYP2A6, a human ortholog of rat CYP2A3, which has been detected in human olfactory mucosa as well as in liver. The CYP2A3 and CYP2A6 cDNAs were cloned into baculovirus, and recombinant viruses were used to produce active enzymes in *Spodoptera frugiperda* (SF9) cells. The metabolic activities of *S. frugiperda* cell microsomal fractions containing CYP2A3 or CYP2A6 were studied in a reconstituted system with purified rabbit NADPH-P450 reductase. CYP2A3 was found to be active toward testosterone, producing 15α-hydroxytestosterone and several other metabolites, but it had only low activity toward coumarin. On the other hand, CYP2A6 was active toward coumarin but not toward testosterone. However, both enzymes were active in the metabolic activation of hexamethylphosphoramide, a nasal procarcinogen, and 2,6-dichlorobenzonitrile (DCBN), a herbicide known to cause tissue-specific toxicity in the olfactory mucosa of rodents at very low doses. In addition, both enzymes were active toward 4-nitrophenol, a preferred substrate for CYP2E1. Consistent with CYP2A3 being a major catalyst in microsomal metabolism of DCBN, the activities of both CYP2A3 and rat olfactory microsomes in DCBN metabolism were inhibited strongly by metyrapone and methoxsalen (ID_{50} <1 μM, with DCBN at 30 μM), but only marginally by 4-methylpyrazole, an inhibitor of CYP2E1. In contrast, the activity of CYP2A6 was only weakly inhibited by metyrapone or methoxsalen (ID_{50} >50 μM). Thus, rat CYP2A3 and human CYP2A6 have differences in substrate specificity as well as tissue distribution. These findings should be taken into account when assessing the risk of exposure to potential nasal toxicants in humans.

Numerous environmental chemicals are known to cause cancer or olfactory neurodegeneration in the nasal cavity in rodents (1, 2). In many cases, the nasal toxicity is tissue specific and is thought to have resulted from metabolic activation of the chemicals in the nasal mucosa by a group of enzymes belonging to the P450 monooxygenase superfamily. Previous studies in rabbits indicated that >10 different P450s are expressed in the olfactory mucosa (3), including two nasal-predominant forms, CYP2A10 and CYP2A11, and an olfactory-specific form, CYP2G1, for which the cDNA structures have been determined (4, 5) and the purified (6) or heterologously expressed (4) proteins have been characterized. Although CYP2G1 is an efficient sex steroid hydroxylase (7), CYP2A10/11 is highly active toward a number of known nasal toxicants, such as *N*-nitrosodiethylamine, HMPA, phenacetin, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and aflatoxin B1 (6, 8, 9). In rats, a species frequently used for toxicity tests and in which most of the olfactory toxicity was observed, the nasal expression of several P450 forms has also been reported (3), including CYP2A3, which is orthologous to rabbit CYP2A10/11. CYP2A3 was first detected in lung and recently found to be predominantly expressed in olfactory mucosa, but it has not been detected in liver or other extrahepatic tissues (10–12). However, although the cDNA and gene structures for this form have been determined (10, 13), catalytically active CYP2A3 has not been obtained; therefore, its role in the metabolic activation and tissue-specific toxicity of olfactory toxicants has not been examined.

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ABBREVIATIONS: P450 or CYP, cytochrome P450; SF9, *Spodoptera frugiperda*; DCBN, 2,6-dichlorobenzonitrile; 4-NP, 4-nitrophenol; 4-MP, 4-methylpyrazole; HMPA, hexamethylphosphoramide; HPLC, high performance liquid chromatography.

In the current study, a cDNA for CYP2A3 was expressed in insect cells lacking endogenous P450 activity, and the substrate specificity of the recombinant cytochrome was characterized. The metabolic activities of rat CYP2A3 toward known nasal toxicants and other P450 substrates were compared with that of human CYP2A6 to determine whether similar metabolic capabilities exist in human nasal mucosa. In addition, the effects of three potential P450 inhibitors, metyrapone, 4-MP, and methoxsalen, on the activities of CYP2A3 and CYP2A6 were examined.

Experimental Procedures

Heterologous expression of CYP2A3 and CYP2A6. The nomenclature used in this report is according to Nelson *et al.* (14). A nearly full-length cDNA clone for CYP2A3 isolated from a rat lung cDNA library was used to construct a full-length 2A3 cDNA through a polymerase chain reaction approach, with use of the Vent DNA polymerase (New England Biolabs, Beverly, MA) and primers (forward primer, 5′-ggaagatctatgctggcctcaggactccttctggtggcctcagtggcc-3′; reverse primer, 5′-gctctagatcaacgggacaagaaactcatag-3′, with the translation initiation codon and stop codon underlined) that contain the 21 nucleotides missing from the 5′ end of the original cDNA as well as linkers with unique restriction sites. The resultant cDNA fragment was ligated into pCR-Script vector (Stratagene, La Jolla, CA); a *Bgl*II/*Not*I fragment was then excised and cloned into the *Bam*HI/*Not*I sites of the baculoviral expression vector pVL1393 (PharMingen, San Diego, CA). Recombinant viruses were made by cotransfecting the transfer plasmid and linearized BaculoGold viral DNA (PharMingen) into SF9 cells. The CYP2A6 expression virus was constructed by Chen *et al.*[1] The cDNA, as an *Eco*RI fragment, was cloned into pBlubac II, and the recombinant virus was constructed using the protocol provided by InVitrogen (San Diego, CA). Details for preparation and titering of virus stocks and P450 expression are provided in Gonzalez *et al.* (15). The recombinant baculoviruses were amplified and used to infect SF9 cells grown in spinner flasks to produce CYP2A3 and CYP2A6. SF9 cells with a cell density of 10^6 cells/ml were infected in TMN-FH media (PharMingen) containing 5% fetal bovine serum with a multiplicity of infection of 10. Hemin was routinely included in the media at 5 μg/ml. Culture of SF9 cells was performed by the Tissue Culture core of the Wadsworth Center (New York State Department of Health, Albany, NY). The cells were harvested at ~72 hr after infection; resuspended in 100 mM Tris-acetate buffer, pH 7.4, containing 1 mM EDTA and 150 mM potassium chloride; and lysed by homogenization with a Brinkmann Polytron (three passes for 10 sec each; Brinkmann Instruments, Westbury, NY). Nuclear fraction and unbroken cells were removed by centrifugation at 1200 rpm in a JA-20 rotor, and the microsomal membrane fraction was obtained as described previously (6). Membrane pellets were resuspended in Tris-acetate buffer (50 mM), pH 7.4, containing 1 mM EDTA and 20% glycerol, and stored at −85°.

Determination of catalytic activities. The contents of reaction mixtures are described in the table or figure legends. The rates of product formation were corrected for zero-time blanks that were quenched before the addition of NADPH. The formaldehyde formed from HMPA *N*-demethylation was measured according to the method of Nash (16). Formation of 4-nitrocatechol from 4-NP was determined as described by Koop (17). Hydroxylation of coumarin was assayed according to the method of Greenlee and Poland (18) with the use of a model LS50B Luminescence Spectrometer (Perkin-Elmer Cetus, Norwalk, CT) in the Biochemistry Core of the Wadsworth Center.

Formation of DCBN-protein adduct was assayed as described recently (19) with use of 2,6-[*ring*-^{14}C]DCBN (16.7 Ci/mol; Sigma Chemical, St. Louis, MO) as a substrate. To determine the rate of DCBN adduct formation, the enzymatic reaction was terminated by addition of 5 volumes of ice-cold acetone. The mixtures were agitated with a vortex instrument and then centrifuged at 2000 × *g* for 10 min. The resulting precipitates were dissolved in 1.0 ml of sodium dodecyl sulfate (1%) and then precipitated with 5 ml of acetone. The sodium dodecyl sulfate/acetone cycle was repeated until no more radioactivity could be extracted and the precipitate from the control incubations did not exhibit any radioactivity. The proteins were then dissolved in 1 M sodium hydroxide. An aliquot of the solution was used for liquid scintillation counting, and the values were compared with those of the total reaction mixture for calculation of the rate of adduct formation. The rates were not corrected for loss of total protein in this procedure, which was <20%.

Testosterone oxidation was assayed as previously described (5, 7). For quantification of testosterone metabolites, the reactions were terminated by the addition of an ice-cold solution of perchloric acid and methanol to final concentrations of 2% and 45%, respectively. The mixture was centrifuged, and an aliquot of the supernatant was analyzed on HPLC. The recovery of labeled steroids in the supernatant fraction was essentially 100%.

Testosterone metabolites were analyzed by HPLC with use of a Waters (Milford, MA) 4-μm Nova-Pak C_{18} column (i.d., 150 × 3.9 mm) preceded by a Nova-Pak C18 Precolumn Cartridge. All analyses were performed at room temperature with a Waters HPLC system consisting of a model 626 pump, a model 996 photodiode array detector, a model 600S controller, and a model 717 plus autosampler and with a Radiomatic model A-500 Radio-Chromatography detector (Packard, Meriden, CT) with a 0.25-ml flow cell. Metabolites were identified on the basis of comigration with available standards.

Other methods and materials. Nasal microsomes were prepared as described previously (20) from the olfactory mucosa of male Wistar rats (200–230 g body weight) from a colony maintained by the Wadsworth Center. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin as the standard. Microsomal P450 was determined according to the procedure of Omura and Sato (21). Solubilization and polyethylene glycol fractionation of CYP2A3- and CYP2A6-containing SF9 cell microsomal preparations (for spectral analysis) were performed as described in previous studies for nasal microsomes (6). Purification of NADPH-P450 reductase from rabbit liver microsomes has been described previously (7). [1,2,6,7-^{3}H(N)]Testosterone was from DuPont-New England Nuclear (Boston, MA). The source of testosterone derivatives has been described previously (6). The preparation and characterization of polyclonal antibodies to rabbit CYP2A10/11 and immunoblotting procedures have been described (20). DNA sequence analysis was performed with an automated DNA sequencer from Applied Biosystems (model 373A; Foster City, CA) by the Molecular Genetics core of the Wadsworth Center. Metyrapone was from Sigma, and methoxsalen was from Aldrich Chemical (Milwaukee, WI). Other materials were obtained as described previously (5–7, 20).

Results

Heterologous expression. The 21 nucleotides missing from the 5′ end of the original CYP2A3 cDNA (10) were replaced based on the sequence of the gene (13) through a megaprimer polymerase chain reaction strategy. The resultant full-length cDNA was inserted into the pVL1393 baculoviral transfer vector, with the ATG start codon of CYP2A3 located 43 bases downstream of the mutated polyhedrin translation initiation codon (22). The integrity of the full-length cDNA was confirmed by sequencing.

The yield of P450 in lysate from recombinant virus-infected SF9 cells ranged from 10 to 40 nmol/liter in different batches

[1] L. Chen, B. W. Penman, J. T. M. Buters, S. Tamura, H. V. Gelboin, J. P. Hardwick, F. J. Gonzalez, and C. L. Crespi, manuscript in preparation.

of cells cultured with addition of hemin. Approximately 50–60% of the cytochrome was found in the microsomal fraction, with ~30% found in the fraction containing unbroken cells and nuclei. The level of the cytochrome in the microsomal fraction ranged from 0.3 to 0.5 nmol/mg of protein, with the expression level of CYP2A3 generally higher than that of CYP2A6.

Spectral analysis. A typical P450 CO-difference spectrum with an absorbance maximum at 448 nm was recorded with detergent-solubilized and polyethylene glycol-fractionated microsomal preparations from CYP2A3- or CYP2A6-expressing SF9 cells, as shown in Fig. 1. P450 spectra were also obtained in crude microsomal fractions from infected cells; however, the spectral peak at ~420 nm, which was probably derived from nonspecific binding of hemin to membrane proteins, as suggested by Buters *et al.* (23), was higher than that shown in Fig. 1. No P450 was detected in microsomal fractions from control cells or cells infected with the wild-type virus (not shown).

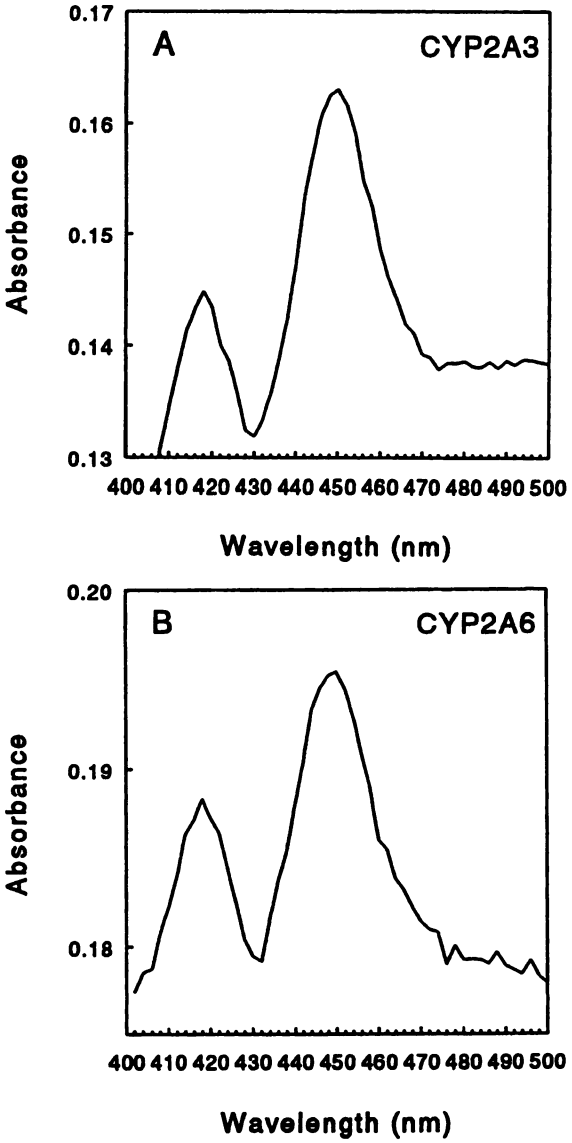

Fig. 1. Spectral analysis of recombinant CYP2A3 and CYP2A6 expressed in SF9 cells. Microsomal preparations from CYP2A3- (A) or CYP2A6- (B) expressing SF9 cells were solubilized with 0.5% cholate and 0.5% Tergitol NP-10 and fractionated with polyethylene glycol. The supernatant fractions after 6% polyethylene glycol precipitation were used directly for P450 measurements without further dilution. Spectra were recorded at 22°.

Immunoblot analysis. Expression of the CYP2A proteins was confirmed by immunoblot analysis with an antibody to CYP2A10/11. As shown in Fig. 2, a single band was detected in the microsomal fractions of rat olfactory mucosa (*lane 1*) and CYP2A3- (*lane 2*) or CYP2A6- (*lane 3*) expressing SF9 cells but not in control cells (*lane 4*). The recombinant CYP2A3 and CYP2A6 migrated to the same position as did rat nasal microsomal CYP2A3 or human liver CYP2A6 (not shown), suggesting that the recombinant mammalian proteins synthesized by SF9 cells are of the same size as their native counterparts and are not post-translationally modified.

Catalytic activities toward coumarin, 4-NP, and testosterone. The activities of CYP2A3, CYP2A6, and rat olfactory microsomes toward three known P450 substrates, coumarin, 4-NP, and testosterone, were determined. These substrates have previously been shown to be metabolized in olfactory microsomes at rates much higher than those found in hepatic microsomes (11, 20, 24). The activities of the recombinant proteins were assayed in a reconstituted system with saturating levels of P450 reductase (with a molar ratio of P450 to reductase of 1:2). Saturation of the reductase was established in experiments with testosterone as a substrate, at ratios of P450 to reductase of 1:1, 1:2, 1:4, and 1:6. Inclusion of dilauroylphosphatidylcholine in the reconstituted system in addition to the lipids present in SF9 cell membrane fractions did not result in an increase in activity (data not given).

As has been reported previously (25), CYP2A6 was active toward coumarin, with a turnover number of 2.14 at a substrate concentration of 0.5 mM (Table 1). However, CYP2A3 had very low activity toward coumarin despite the high activity of rat olfactory microsomes toward this substrate. In experiments not presented, CYP2A6 and olfactory microsomes were found to have apparent K_m values of 6 and 15 μM, respectively, for coumarin hydroxylation. However, an apparent K_m value for CYP2A3 could not be obtained because of its low activity with this substrate.

Both CYP2A3 and CYP2A6 were active toward 4-NP, a preferred substrate for CYP2E1, with the turnover number of CYP2A6 being more than twice as high as that of CYP2A3 but only approximately one third of that found in rat olfactory microsomes. However, subsequent kinetic analysis (not shown) indicated that all three enzyme preparations had

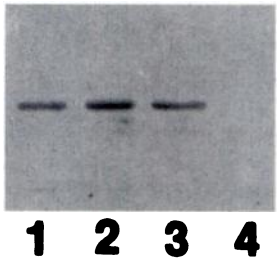

Fig. 2. Immunoblot analysis of recombinant CYP2A3 and CYP2A6. Rat olfactory microsomes (*lane 1*) or microsomal preparations from SF9 cells infected with baculovirus harboring CYP2A3 (*lane 2*) or CYP2A6 (*lane 3*) cDNA or from uninfected SF9 cells (*lane 4*) were analyzed on immunoblots (10 μg of protein/lane) with a polyclonal anti-CYP2A10/11 antibody.

TABLE 1

Metabolism of testosterone, coumarin, and 4-NP by CYP2A3, CYP2A6, and rat olfactory microsomes

Reaction mixtures contained 100 mM phosphate buffer, pH 7.4 (for testosterone and coumarin metabolism) or 6.8 (for 4-NP hydroxylation), a substrate at concentrations as indicated in parentheses, and 0.04 (for testosterone hydroxylation) or 0.1 μM (for coumarin and 4-NP hydroxylation) olfactory microsomal P450 (0.31 nmol of P450/mg of microsomal protein) or recombinant CYP2A3 (0.52 nmol of P450/mg of protein) or CYP2A6 (0.37 nmol of P450/mg of protein) reconstituted with purified P450 reductase. For testosterone metabolism, [1,2,6,7,^{3}H(N)]-testosterone (2.5 Ci/mmol) was used, and the mixture also contained 1 mM L-ascorbic acid. The reactions were initiated by the addition of NADPH to a final concentration of 1 mM and carried out at 37° for 3–10 min, during which product formation was linear with time. In control reactions, the microsomal fractions from uninfected SF9 cells (0.18 mg of protein/ml) were used.

Enzyme preparation	Rate of product formation[a]		
	Coumarin 7-hydroxylation (0.5 mM)	4-NP hydroxylation (0.2 mM)	Testosterone hydroxylation[b] (10 μM)
CYP2A3	0.21 ± 0.02	1.52 ± 0.10	1.45 ± 0.05
CYP2A6	2.14[d]	3.75 ± 0.60	0.12 ± 0.02[c]
Olfactory microsomes	3.09 ± 0.25	10.55 ± 3.06	12.4[d]
Control cells[e]	<0.01	0.15 ± 0.05	0.04 ± 0.01

[a] Unless indicated otherwise, values are mean ± standard deviation (n = 3 or 4) and are expressed as nmol/min/nmol of P450.
[b] The combined rates of formation of all metabolites (as shown in Fig. 4) are reported.
[c] 0.04 ± 0.01 nmol/min/mg of protein, which is identical to the value obtained with control SF9 cells; the product was androstenedione.
[d] Values reported are the average of two determinations, with differences <10% of the mean.
[e] Expressed as nmol/min/mg of protein.

similar apparent K_m values (88, 63, and 73 μM for CYP2A3, CYP2A6, and olfactory microsomes, respectively). The 4-NP hydroxylase activity was determined at pH 6.8, an optimal pH for CYP2E1 (17), although the activities determined at pH 7.4 were not significantly different (data not given). In other experiments not presented, the addition of purified cytochrome b_5 (at a molar ratio of P450 to cytochrome b_5 of 1:4) decreased the activity of CYP2A3 by ~50%.

In contrast to the low activity toward coumarin, CYP2A3 was quite active toward testosterone, generating multiple metabolites with a turnover number for total product formation of 1.45 at a substrate concentration of 10 μM. However, CYP2A6 was not active toward testosterone. The lack of activity of CYP2A6 in testosterone hydroxylation has also been reported by others at higher substrate concentrations (26, 27). Interestingly, the activity of rat olfactory microsomes was very high toward testosterone, with a turnover numbers of 12.4, approximately nine times as high as that of recombinant CYP2A3. In other experiments not presented, the addition of purified cytochrome b_5 at a P450-to-cytochrome b_5 ratio of 1:4 resulted in a 50% inhibition of the activity of CYP2A3 toward testosterone, similar to that seen with 4-NP as a substrate.

The HPLC profiles of testosterone metabolites generated in reactions with CYP2A3 or olfactory microsomes are shown in Fig. 3. Five metabolite peaks were detected in reaction mixtures with CYP2A3 (Fig. 3A), and the same metabolite peaks as well as an additional peak with a retention time (30.2 min) longer than that of testosterone (27.9 min) were detected in olfactory microsomal reactions (Fig. 3C). Formation of these metabolites was NADPH dependent (data not given) and was not observed with reconstituted microsomal fractions from control (Fig. 3B) or CYP2A6-expressing SF9 cells (not shown). However, the formation of a metabolite (designated A) with a retention time of ~27 min, which comigrated with androstenedione, was not NADPH dependent (data not given) and was also observed in reactions with control SF9 cells (Fig. 3B). Based on comparisons with the retention time of available standards of testosterone derivatives, the three major NADPH-dependent products were identified as 15β-, 15α-, and 2β-hydroxytestosterone, respectively, but the two minor products, P3 and P5, as well as P6, did not comigrate with any of the available standards, including 2α-, 2β-, 6α-, 6β-, 7α-, 11α-, 11β-, 14α-, 15α-, 16α-, 16β-, and 19-hydroxytestosterone; 11-keto- and 16-keto-testosterone; and androstenedione. The peak eluted in the void volume represents nonenzymatic degradation products of radiolabeled testosterone.

Catalytic activities toward olfactory toxicants. The activities of the recombinant CYP2A3 and CYP2A6 toward HMPA, a known nasal carcinogen in rats, and DCBN, an olfactory-specific toxicant, were examined and compared with those of rat olfactory microsomes. The kinetic parameters of HMPA demethylase activity are shown in Table 2. The K_m and V_{max} values were determined from Lineweaver-Burk plot analysis with substrate concentrations in the range of 0.5–4 mM. Both CYP2A3 and CYP2A6 were active toward HMPA, with comparable apparent catalytic efficiencies, as indicated by the V_{max}/K_m ratio. However, a 10-fold greater catalytic efficiency was found with olfactory microsomes, resulting from a lower apparent K_m and a higher apparent V_{max} value.

The turnover numbers for the formation of DCBN-protein adducts from DCBN are shown in Table 3. Both CYP2A3 and CYP2A6 were active toward DCBN, with turnover numbers for CYP2A3 being approximately twice as high as that of CYP2A6. However, the activity of CYP2A3 in the reconstituted system was only approximately one half of that found in olfactory microsomes. No activity was detected in control SF9 cells.

Inhibition of the metabolic activation of DCBN. The inhibitory effects of two compounds, metyrapone and methoxsalen, on DCBN-protein adduct formation were examined with CYP2A3 and CYP2A6 in a reconstituted system and with rat olfactory microsomes. As shown in Fig. 4, metyrapone and methoxsalen are highly inhibitory toward the activity of CYP2A3 and of nasal microsomes; with substrate present at 30 μM, a 50% inhibition was achieved with the inhibitors at ~≤1 μM. However, the inhibitory effects of the two compounds were much weaker toward CYP2A6; the extent of inhibition at the highest dose (50 μM) examined was <50%. In experiments not presented, 4-MP, a well-established inhibitor of CYP2E1, also had weak inhibition of the activities of CYP2A3 and olfactory microsomes, with the extent of inhibition at 50 μM being <40%, but it did not inhibit the activity of CYP2A6.

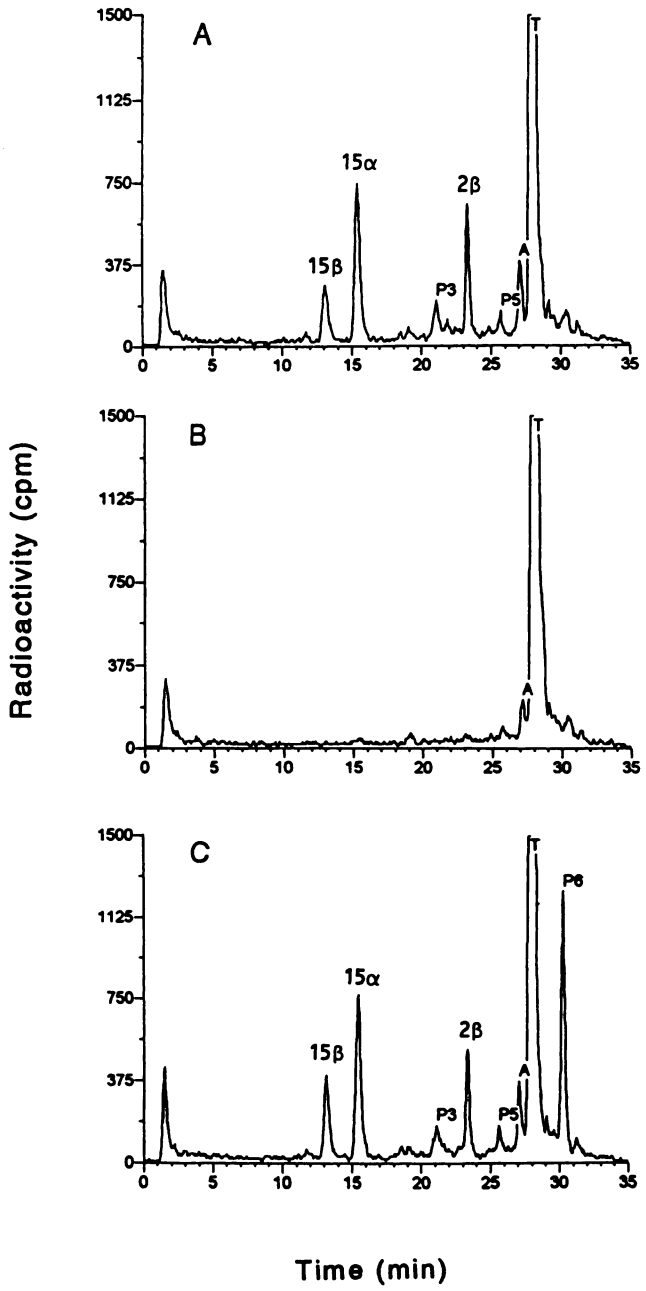

Fig. 3. HPLC profiles of testosterone metabolites generated by recombinant CYP2A3 and rat olfactory microsomes. Testosterone hydroxylase assays were carried out as described in the legend to Table 1. Product peaks, including 15β-, 15α-, and 2β-hydroxytestosterone, and three unidentified products, designated P3, P5, and P6, as well as the substrate (*T*) and androstenedione (*A*) are labeled. The metabolites and the parent compound extracted from reactions with CYP2A3 (A), control SF9 cells (B), and olfactory microsomes (C) were separated by reverse-phase HPLC with an on-line radioactivity detector as described in Experimental Procedures.

Discussion

The size of the olfactory epithelium of rodents is inadequate for attempts to isolate P450 forms for characterization, although highly purified nasal P450s have been obtained from rabbits in our previous studies (6). Thus, in the current study, a baculoviral expression system was used to obtain CYP2A3. The heterologously expressed enzyme has a typical P450 spectrum and the same molecular weight as that of the native olfactory microsomal CYP2A3 and is active toward a number of substrates when reconstituted with NADPH-P450 reductase. The substrate specificity of CYP2A3 was compared with that of CYP2A6, a human ortholog of the rat P450 form. The regulation and function of P450s of the CYP2A subfamily were recently reviewed (28). The catalytic activities of CYP2A6 have been studied by a number of groups with enzyme preparations purified from human liver (26) or obtained from different heterologous expression systems (28). Known substrates for CYP2A6 include many toxicants and procarcinogens, such as *N*-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 4,4′-methylene-bis(2-chloroaniline), *N*-nitrosodimethylamine, N-nitrosodiethylamine, aflatoxin B1, benzo[a]pyrene, 6-aminochrysene, 2-amino-3-methylimidazo[4,5-f]quinoline, and 2-amino-3,5-dimethylimidazo[4,5-f]quinoline (28). In addition, CYP2A6 is the major coumarin hydroxylase in liver microsomes (28). A genetic polymorphism in the *CYP2A6* gene has also been described in which up to 1% of Caucasians and possibly a higher percentage of Asians lack expression (29).

The abilities of heterologously expressed CYP2A3 and CYP2A6 to activate nasal toxicants, including HMPA and DCBN, were examined in the current study because both P450 forms are expressed in the olfactory mucosa (12, 30). HMPA is an important industrial solvent that causes nasal tumors in rats at concentrations as low as 50 ppb through inhalation exposure for 2 years (31). Previous studies have indicated that HMPA is metabolized by P450 to formaldehyde (1), which is also a nasal carcinogen. Studies with rabbit nasal P450s indicated that CYP2A10/11 plays an important role in the metabolic activation of HMPA (6, 20), and a recent study indicated that human CYP2A6 expressed in lympho-

TABLE 2

Kinetic parameters of HMPA metabolism by CYP2A3, CYP2A6, and rat olfactory microsomes

Reaction mixtures contained 50 mM phosphate buffer, pH 7.4; HMPA at concentrations ranging from 0.5 to 4 mM; and various P450 preparations as described in the legend to Table 1. The reactions were carried out at 37° for 10 min, during which product formation was linear with time. The formation of formaldehyde from HMPA was determined as described in Experimental Procedures. The values reported are derived from Lineweaver-Burk plot analysis of averaged turnover numbers of triplicate determinations with differences <10% of the mean.

Enzyme preparation	K_m	V_{max}	V_{max}/K_m
	mM	*nmol/min/nmol of P450*	
CYP2A3	1.6	10.8	6.8
CYP2A6	2.9	17.3	6.0
Olfactory microsomes	0.6	45.3	75.5

TABLE 3

Metabolic activation of DCBN by CYP2A3, CYP2A6, and rat olfactory microsomes

Reaction mixtures contained 50 mM phosphate buffer, pH 7.4; 30 μM 2,6[*ring*-^{14}C]DCBN; and various P450 preparations as described in the legend to Table 1. The reactions were carried out at 37° for 10 min, during which product formation was linear with time. The formation of DCBN-protein adduct from DCBN was determined as described in Experimental Procedures. Values are mean ± standard deviation (n = 3).

Enzyme preparation	Rate of product formation
	pmol/min/nmol of P450
CYP2A3	178.8 ± 18.8
CYP2A6	87.4 ± 18.5
Olfactory microsomes	354.3 ± 21.7
Control SF9 cells	None detected

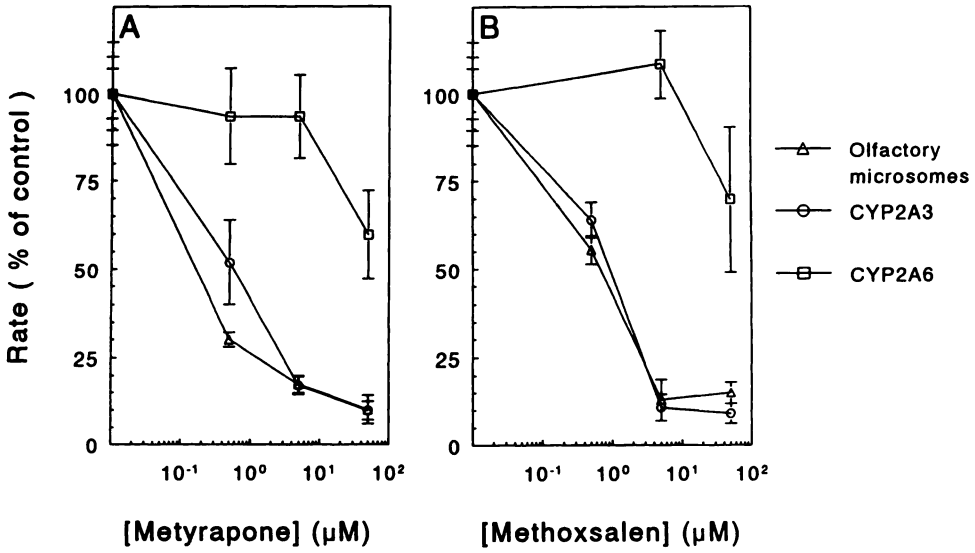

Fig. 4. Effects of metyrapone and methoxsalen on DCBN-protein adduct formation. The activities of recombinant CYP2A3, CYP2A6, or rat olfactory microsomes in the formation of DCBN-protein adducts were determined with metyrapone (A) or methoxsalen (B) added at 0, 0.5, 5, or 50 μM. Contents of reaction mixtures and the assay procedures were the same as described in the legend to Table 3 for DCBN metabolism. Values are mean ± standard deviations (*error bars*) from three experiments. The concentration of inhibitors is shown in logarithmic scale, and the activities are shown as a percentage of control rates determined in the absence of an inhibitor.

blastoma cells is active toward HMPA as well.[2] However, the forms that are responsible for the metabolic activation of HMPA in rats, in which carcinogenicity was demonstrated, have not been identified previously. Results from this study indicate that CYP2A3, a nasal-predominant P450, is active in the metabolic activation of HMPA *in vitro*. Furthermore, CYP2A3-catalyzed HMPA metabolism is inhibited by metyrapone, which has been shown to inhibit microsomal HMPA metabolism and HMPA-induced DNA-protein cross-link (32). These results are consistent with CYP2A3 being the major form responsible for the metabolic activation of HMPA in nasal tissue. The role of CYP2A3 in microsomal metabolism of HMPA will be further determined when specific inhibitory antibodies to this form become available. In addition, CYP2A6 expressed in SF9 cells is also active toward HMPA, with an apparent catalytic efficiency similar to that of CYP2A3, which suggests that HMPA has the potential to cause toxicity in human nasal mucosa.

CYP2A3 and CYP2A6 were also found to be active in the metabolic activation of DCBN. The herbicide DCBN (dichlobenil) is a potent, olfactory-specific toxicant in rats and mice (33, 34). In a recent study from this laboratory, DCBN was found to be metabolized by rat and rabbit olfactory microsomal P450s to a reactive intermediate, 2,3-oxo-DCBN, which is subsequently converted to DCBN-protein adducts as well as other products (19). Furthermore, lymphoblastoma cells containing heterologously expressed human CYP2A6 and CYP2E1, but not CYP1A2, were active in the metabolism of DCBN. The turnover number of CYP2A6 in the formation of DCBN-protein adducts determined in the previous experiment with P450s expressed in human lymphoblastoma cells is similar to that obtained in the current study with the P450 expressed in SF9 cells, confirming that human CYP2A6 is capable of metabolic activation of the potential olfactory toxicant.

The activities of CYP2A3 as well as those of olfactory microsomes in the formation of DCBN-protein adducts were strongly inhibited by metyrapone and methoxsalen. In previous studies by others (33), metyrapone was found to inhibit DCBN metabolism in rat olfactory microsomes *in vitro* and to prevent DCBN toxicity *in vivo*. This is consistent with the current finding that metyrapone inhibits DCBN metabolism by CYP2A3 and indicates that the preferential expression of CYP2A3 in the olfactory mucosa may play a major role in the tissue-specific activation and toxicity of DCBN. The effects of 4-MP, a known inhibitor of CYP2E1, on DCBN metabolism were also examined. Previous studies indicated that CYP2E1 is also active toward DCBN (19); however, indirect evidence suggested that CYP2E1 may not play a major role in olfactory microsomal metabolism of DCBN (24). In the current study, 4-MP very weakly inhibited the activity of CYP2A3 or olfactory microsomes toward DCBN, which apparently precludes CYP2E1 from playing a role in the tissue-specific toxicity of DCBN. However, it remains to be determined whether this P450 form will contribute to olfactory DCBN metabolism after induction by alcohol or other xenobiotic compounds. Interestingly, the activity of CYP2A6 was only weakly inhibited by metyrapone or methoxsalen and was not inhibited by 4-MP, revealing subtle differences in the substrate binding sites of CYP2A3 and CYP2A6.

The substrate specificity of the two orthologous P450 forms was further characterized with the use of known P450 substrates, including coumarin, testosterone, and 4-NP. Coumarin and testosterone have been used previously to distinguish structurally similar P450 forms in the CYP2A subfamily. For example, in mice, CYP2A4 is active toward testosterone but not toward coumarin, whereas CYP2A5 is active toward coumarin but not toward testosterone (35, 36). In rabbits, CYP2A10, but not CYP2A11, is active toward coumarin; neither form has high activity toward testosterone (5). The current data indicate that, more like CYP2A4, CYP2A3 is not very active in coumarin 7-hydroxylation but is highly active in testosterone hydroxylation, and, consistent with previous reports, CYP2A6 is active toward coumarin but inactive toward testosterone (26, 27). A previous report by Lindberg and Negishi (37) indicated that alteration of amino acid residues at position 117, 209, or both results in switching of substrate specificity toward coumarin and testosterone between CYP2A4 and CYP2A5. The amino acid residue at position 209 is a phenylalanine for both CYP2A3 and CYP2A6 (10,

[2] J. R. Thornton-Manning, J. A. Hotchkiss, K. D. Rohrbacher, X. Ding, and A. R. Dahl, unpublished observations.

25), as is in CYP2A5, the coumarin hydroxylase (36), whereas the residue at position 117 is a valine in CYP2A6, as in CYP2A5, but an alanine in CYP2A3, as in CYP2A4, the testosterone hydroxylase (35). Thus, it would be interesting to determine whether the amino acid residue at position 117 also plays a critical role in conferring the different substrate specificity to CYP2A3 and CYP2A6.

The olfactory mucosa has very high activities toward sex steroids. Previous studies from this laboratory indicated that rabbit CYP2G1, an olfactory-specific form, is an efficient steroid hydroxylase (7). Immunochemical studies with antibodies to CYP2G1 indicated that this form plays a major role in the olfactory microsomal metabolism of testosterone, estradiol, and progesterone in rabbits. In rats, the activity of CYP2G1 toward steroid compounds is not known because a catalytically active form has not been obtained. However, the current finding that CYP2A3 has high activity toward testosterone, producing most of the metabolites found in microsomal reactions, suggests that this olfactory-predominant protein may also play an important role in steroid metabolism in this tissue. The relative importance of the two forms in microsomal steroid metabolism remains to be determined when heterologously expressed CYP2G1 and specific antibodies or chemical inhibitors to these enzymes become available.

CYP2A3 is also active toward 4-NP, a substrate that has been used to monitor CYP2E1 activity in microsomal reactions. However, 4-NP hydroxylation by CYP2A3 differs from that by CYP2E1 in that although the activity of CYP2E1 was approximately twice as high at pH 6.8 as at pH 7.4 (17), the activity of CYP2A3 was not significantly affected by similar changes in pH. In addition, the activity of CYP2E1 was stimulated by the addition of cytochrome b_5, whereas that of CYP2A3 was slightly inhibited. However, even though the turnover number of CYP2A3 toward 4-NP is lower than that reported for CYP2E1 (17), the former enzyme, which is predominant in the olfactory tissue, may contribute significantly to 4-NP hydroxylase activity in olfactory microsomes. Therefore, 4-NP hydroxylase activity may not be a good indicator for the level of CYP2E1 in olfactory microsomes, where CYP2E1 is a minor form (3). In this connection, CYP2A6 had an apparent K_m similar to that of CYP2A3 toward 4-NP and a turnover number twice as high as that of CYP2A3 and may therefore similarly contribute to the metabolism of 4-NP in experiments with human microsomes.

It is not clear why the activities of CYP2A3 in the reconstituted system were always lower than those found with olfactory microsomes. The addition of purified cytochrome b_5 or dilauroylphosphatidylcholine did not increase the activities. It is possible that a portion of CYP2A3 may have formed stable complexes with proteins in the SF9 cell membrane preparation and thus may not be available to complex with added P450 reductase. Alternatively, components of nasal microsomal membranes may be required for optimal reconstitution of CYP2A3 activity, as suggested by a recent study with CYP3A4 (38) in which higher rates of substrate hydroxylation by CYP3A4 were achieved by using membrane vesicles made from microsomal phospholipids. Further studies to clarify these issues with purified CYP2A3 are warranted.

In conclusion, the current study with heterologously expressed CYP2A3 supports our hypothesis that this olfactory-predominant form plays an important role in the tissue-specific toxicity of two known nasal toxicants, HMPA and DCBN. The finding that human CYP2A6, expressed in SF9 cells, is also active toward these compounds confirms previous observations with CYP2A6 expressed in lymphoblastoma cells and suggests potential risk of olfactory toxicity associated with human exposure to these xenobiotics. Comparisons of the substrate specificity and inhibitor selectivity of CYP2A3 with those of CYP2A6 indicated that the two orthologous forms may have subtle differences in their substrate-binding sites, which should be taken into consideration when assessing the risks of exposure to other potential nasal toxicants in humans. Furthermore, these studies also indicate that the role of the *CYP2A6* polymorphism (29) in nasal cancer susceptibility should be investigated.

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