Kimberly C. Day, Charles G. Plopper and Michelle V. Fanucchi *Am J Physiol Lung Cell Mol Physiol* 291:75-83, 2006. First published Feb 3, 2006; doi:10.1152/ajplung.00356.2005

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Age-specific pulmonary cytochrome *P*-450 3A1 expression in postnatal and adult rats

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Submitted 16 August 2005; accepted in final form 30 January 2006

Day, Kimberly C., Charles G. Plopper, and Michelle V. Fanucchi. Age-specific pulmonary cytochrome P-450 3A1 expression in postnatal and adult rats. Am J Physiol Lung Cell Mol Physiol 291: L75-L83, 2006. First published February 3, 2006; doi:10.1152/ajplung.00356.2005.—A major cause of death and illness in children under the age of five, most living in polluted cities, is respiratory disease. Previous studies have shown that neonatal animals are more susceptible to bioactivated pulmonary cytotoxicants than adults, despite lower expression of the pulmonary cytochrome P-450s (CYP450s) thought to be involved in bioactivation. One CYP450 that is well documented in the bioactivation of many drugs and environmental toxicants in adult lung, but whose expression has not been evaluated during postnatal pulmonary development, is CYP450 3A (CYP3A). We compared age-specific expression of CYP3A1 in 7-day-old and adult male Sprague-Dawley rats. Unlike those shown for previously studied pulmonary CYP450s, expression levels for CYP3A1 mRNA in differentiating airway cells of postnatal rats are the same as in fully differentiated airway cells of adults. CYP3A1 protein expression (28%) and enzymatic activity (23%) were lower in postnatal airways compared with adults. Although other CYP450 immunoreactive proteins are primarily expressed in nonciliated cells, immunoreactive CYP3A1 protein was expressed in both ciliated and nonciliated cells in postnatal and adult rat proximal airways. CYP3A1 protein is detected diffusely throughout ciliated and nonciliated cells in 7-day-old rats, whereas it is only detected in the apex of these cells in adult rats. This study demonstrates that the lungs of postnatal rats have detectable levels of CYP3A1 and that CYP3A1 mRNA expression appears not to be age dependent, whereas steady-state CYP3A1 protein levels and enzyme activity show an age-dependent pattern.

pulmonary development; cytochrome P-450 3A

RESPIRATORY DISEASES ARE ONE of the five major causes of death and illness in children under the age of five (69). Exposure to environmental pollutants may make a significant contribution to respiratory disease. Children are disproportionately exposed to environmental pollutants compared with adults because of factors such as elevated levels of exposure, immature metabolic pathways, and developing organ systems that are easily disrupted. These factors may contribute to the heightened susceptibility and place children at a greater risk than adults (31). The cytochrome *P*-450 (CYP450) monooxygenase system consists of enzymes that are responsible for phase I metabolism of many environmental contaminants, therapeutic drugs, and endogenous compounds such as steroid hormones (64). Developmental expression of CYP450 monooxygenases in lung and liver of humans and rodents begins during the fetal stage and continues to increase through infancy and well into childhood (4, 9, 11, 18). Therefore, there is a disparity in metabolism of xenobiotics and drugs between children and adults (for comprehensive reviews see Refs. 20 and 21). For example, a dose of acetaminophen that causes toxicity in adults has no toxic effect in children because of lower metabolic activation of acetaminophen (35, 54). In contrast, cisapride, used to treat gastroesophageal reflux with no adverse effects in adults, causes cardiac dysrhythmias when given to neonates and infants because of an inability to oxidize cisapride (63). Furthermore, neonatal and postnatal rodents are more susceptible than adults to lung injury from air pollutants such as diesel exhaust and environmental tobacco smoke, components of which are metabolized by CYP450 monooxygenases (12, 13, 15, 17, 18, 25, 32). Several factors may explain the differences in susceptibility and toxicity from exposure to xenobiotics such as 1-nitronaphthalene and 4-ipomeanol between the postnatal and adult age groups (3, 40, 50, 55). One contributing factor appears to be the apparent mismatch between CYP450 isozyme activity levels involved in metabolic activation of xenobiotics. Another factor may be that more than one CYP450 isozyme has the potential to activate these xenobiotics and that some of these isozymes may be more highly expressed in children than adults.

For many species, including humans, several of the CYP450 isozymes detected in the nonciliated cells of the lung (2B, 4B, 2F, 1A1, 2J, and the *P*-450 reductase) are not expressed or are expressed at very low levels at birth. The developmental expression of individual CYP450 isozymes is species specific and site specific within the lung, with postnatal species having lower detectable pulmonary steady-state mRNA, protein, and enzyme activity (9, 11, 16, 23, 24, 32, 47, 49). These cell populations differentiate postnatally in rabbit, rats, and mice, and the differentiation occurs in a proximal-to-distal pattern (14, 23, 24, 49, 50, 60). Increases in CYP450 expression occur in parallel with the differentiation of the epithelium.

CYP450 subfamily 3A (CYP3A) has been found in the respiratory system of several species, including human (1, 53) and rat (51). CYP3A metabolizes a wide range of structurally diverse compounds that include xenobiotics, clinical drugs, and steroid hormones (8, 37, 39). Examples of commonly metabolized compounds include polycyclic aromatic hydrocarbons, erythromycin, cisapride, verapamil, codeine, cyclosporin A and G, acetaminophen, diazepam, and progesterone (37). In humans, bioactivation of air pollutants, particularly polycyclic

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hydrocarbons, may be mediated through the CYP3A isozyme (1, 8, 22, 38). In humans, CYP3A is found in several pulmonary cell populations, including bronchial ciliated and mucous cells, bronchiolar epithelium, type I and type II alveolar epithelium, endothelium, and alveolar macrophages (1). Little is known about the pattern of developmental expression of pulmonary CYP3A in humans, and less is known about its expression in rodents. Because rodents are often used as models for human drug/toxicology studies, the purpose of this study was to determine whether there are age-specific differences in expression of CYP3A1 in 7-day-old (postnatal) and adult rats. This study addresses the following questions: 1) Are CYP3A1 mRNA, protein, and enzymatic activity expressed in the lungs of postnatal rats? 2) Does expression of CYP3A1 mRNA, protein, and enzymatic activity in postnatal rats differ from that in adult rats?

MATERIALS AND METHODS

Animals. Specific-pathogen-free adult male Sprague-Dawley rats (250–300 g) and lactating female rats with male pups (7 days old) were obtained from Harlan (San Diego, CA). Animal protocols were reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee. Seven days was chosen as an early postnatal time point to allow comparisons with previously published studies (13, 17, 18, 23–25, 60). All animals were allowed free access to food and water and housed in laminar flow hoods in Association for Assessment and Accreditation of Laboratory Animal Care International-approved animal facilities on a 12:12-h light-dark cycle at the University of California, Davis. After anesthetization with pentobarbital sodium (100 mg/kg) and exsanguination, the trachea was exposed and cannulated.

CYP3A1 mRNA expression: laser capture microdissection. Lungs from five postnatal and five adult rats were removed from the chest cavity, inflated with ice-cold Zinc-fix (57), and infiltrated for 4 h at 4°C. The left lobe was removed, cut perpendicular to the main axial airway pathway, and embedded into paraffin. RNase-free solutions were used to deparaffinize and dehydrate 7-µm-thick sections, which were visualized with a $\times 20$ objective and bright-field illumination. The epithelia at the most proximal airway (the bronchus) and the most proximal blood vessel (pulmonary vein including both smooth muscle and cardiac muscle) were laser captured with a PixCell III system (Arcturus, Mountain View, CA). A 7.5-µm-diameter laser was used at an intensity of 225, a power of 60 mW, and duration between 0.75 and 1 s. Approximately 3,500-6,000 pulses were captured onto plastic caps from four sections per animal. These caps were incubated for 30 min at 42°C and then briefly centrifuged to collect cell extract. RNA was isolated according to the manufacturer's standard protocol for PicoPure CapSure Macro LCM Caps RNA isolation (Arcturus). RNA was eluted with 11 μ l of elution buffer and stored at -80° C until RNA was reverse transcribed.

Real-time quantitative PCR. All RNA reverse transcription and PCR reactions were performed with TaqMan reagents (Applied Biosystems, Foster City, CA) unless otherwise indicated and processed

according to manufacturer's instructions. Primer and probe sequences were designed with Primer Express software (Applied Biosystems) and are listed in Table 1. RNA was reverse transcribed in a 20-µl reaction mixture containing $1 \times$ TaqMan RT Buffer, 5.5 mM MgCl₂, 500 µl of dNTPs, 2.5 µl of random hexamers, 0.4 U/µl ribonuclease inhibitor, and 1.25 U/µl multiscribe reverse transcriptase. Reaction mixtures were incubated at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Individual PCR reactions contained $1 \times$ TaqMan Mastermix, 4.54 µl of cDNA, 400 nM 5' and 3' primers, and 100 nM probe. PCR reactions were performed with the following protocol: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene.

Results were calculated based on modifications from methods previously described (36, 43, 44) and reported as the relative expression ratio of a target gene (rat CYP450 3A1) to an internal reference gene (GAPDH). Briefly, the threshold cycle, C_t, is defined as the point at which the first significant increase in fluorescence is observed. The slope (*S*) was determined for each individual PCR run from serial RNA dilutions and used to calculate real-time PCR efficiencies (E) for target and reference genes with the equation $E = 10^{-1/S}$. The relative expression ratio is described by the equation ratio = $E_{3A1}^{dC_1, 3A1}$ gene $/E_{GAPDH \text{ gene}}^{dC_1, GAPDH \text{ gene}}$ (43). dC_t is defined for each sample as the difference between the reference calibrator gene and test gene [C_{t(reference calibrator)} - C_{t(CYP450 3A1)} or C_{t(GAPDH)}]. The reference calibrator sample used in this study was adult rat isolated airway RNA.

Gel electrophoresis and immunoblotting. Lungs from four postnatal and four adult rats were removed from the chest cavity, inflated with 1% SeaPlaque agarose (BioWhittaker Molecular Applications, Rockford, ME) in 1% Waymouth MB 752/1 medium (Sigma, St. Louis, MO) at 37°C, and placed into ice-cold Waymouth MB 752/1 medium for 15 min. The airway tree was separated by blunt dissection as previously described (46). Adult and postnatal samples were placed into 1.0 or 0.5 ml of lysis buffer (68), respectively, for at least 1 h. All samples were homogenized and centrifuged at 9,000 g for 15 min, and the supernatant was stored at -80°C until analysis. A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine protein concentration. Protein samples were mixed with an 8× loading buffer (30), boiled for 5 min, and then stored at -20° C until gel loading. For all samples, 70 µg of protein were loaded onto a 10% precast mini polyacrylamide ready gel Tris·HCl (Bio-Rad). To generate a standard curve, five internal protein concentrations (1, 17, 35, 53, and 70 µg) of 9,000 g adult rat liver protein were loaded onto each individual gel. Gels were electrophoresed on a Mini Protein II (Bio-Rad) for 60 min with 155 V and then transferred to a polyvinylidene difluoride membrane for 60 min with 100 V. Membranes were blocked with a 5% nonfat dry milk solution made in Tris-buffered saline containing 0.1% Tween 20 (TBST). To detect CYP3A1 immunoreactive protein, membranes were incubated with the anti-rat CYP3A1 (AB1253, Chemicon, Temecula, CA) antibody for 1 h on a rotator at room temperature and then for 21 h at 4°C. Membranes were rinsed several times with TBST before fluorescence secondary antibody Cy5-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was applied for 1 h. Gels were scanned on a

Table 1. Primers/probes used for TaqMan real-time RT-PCR analysis

Gene Name	Primer/Probe Sequence
Cytochrome <i>P</i> -450 3A1 Glyceraldehyde-3-phosphate-dehydrogenase	Forward:CAGCAGCACACTTTCCTTTGTC Reverse:CTCCTCCTGCAGTTTCTTCTGTGTA Probe:(6-FAM)TGCATTCCCTGGCCACTCACCC(TAMRA) Forward:TGTGTCCGTCGTGGATCTGA Reverse:CCTGCTTCACCACCTTCTTGA Probe:(6-FAM)CGCCTGGAGAAACCTGCCAAGTATG(TAMRA)

Typhoon 8600 with a 633-nm laser and analyzed with ImageQuant 5.1 software (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). Signal intensity was quantified according to previously published methods (2) for each sample (n = 4) based on linear regression analysis from a standard curve that was generated from internal standards and graphed to represent mean (SD) immunoreactive protein expression intensity. To validate that the amount of protein loaded was consistent between adult and 7-day-old samples, we identified the actin protein and compared the relative densities for the protein with the same loading conditions (70 µg). As a secondary/loading control, actin was identified with an anti-rat actin antibody (A2668, Sigma), and blots were scanned and analyzed as described above. Relative expression values for the actin loading control were not different between adult [191.92 (SD 8.37)] and 7-day old [206.21 (SD 9.68)] rats.

Immunohistochemistry. Lungs from five postnatal and five adult rats were inflated in the chest cavity via tracheal cannula with 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in a 0.1 M phosphate buffer, pH 7.4, at 30 cm of pressure for 1 h. The left lobe was removed, cut perpendicular to the main axial airway pathway, and embedded into paraffin. Paraffin sections (6 µm thick) were analyzed for site-specific immunoreactive labeling of CYP3A1 protein in the proximal and distal airways with the same primary antibody that was used for immunoblotting. After overnight incubation at 4°C with primary antibody, immunoreactive protein was visualized with a Vectastain Peroxidase Rabbit IgG ABC kit (Vector Laboratories, Burlingame, CA) using nickel and 3,3'-diaminobenzidine tetrahydrochloride (Sigma). To determine the optimal primary antibody concentration, serial dilutions were done. A negative control was included in each run by substituting phosphate-buffered saline for primary antibody. Images of the most proximal airways and corresponding pulmonary veins were taken with an Olympus O-Color 3 (QImaging, Burnaby, BC, Canada) camera attached to an Olympus BX-41 microscope connected to a personal computer. Images were compiled in Adobe Photoshop.

Microsomal activity of CYP3A1 assay. Lungs from 40 postnatal rats and 12 adult rats were removed from the chest cavity. Bluntdissected airways from adult and postnatal rats were pooled and placed into ice-cold Waymouth MB 752/1 medium. Livers were removed and pooled from postnatal rats, and individual livers were removed from adult rats and placed into ice-cold Waymouth MB 752/1 medium. Samples were homogenized on ice with a Potter-Elvehjem-style homogenizer with a Teflon pestle in a buffer consisting of 20 mM Tris, pH 7.4, 150 mM KCl, 0.2 mM sodium EDTA, 0.5 mM dithiothreitol, and 15% glycerol. The mixture was then centrifuged at 9,000 g at 4°C for 20 min. Microsomal pellets were recovered by ultracentrifugation (100,000 g for 60 min), and pellets were resuspended in 0.1 M potassium phosphate, pH 7.4. A Bradford protein assay was used to determine microsomal protein concentration. The amount of CYP3A1 activity was based on the formation of midazolam metabolites 1-hydroxymidazolam (1-OH MDZ) and 4-hydroxymidazolam (4-OH MDZ) (Gentest, Woburn, MA). The assay and HPLC conditions as described below were modified from published methods (19, 28, 65). Each assay had a final volume of 100 µl of 0.1 M potassium phosphate buffer, pH 7.4, and contained 0.3 mg microsomal protein, 250 µM midazolam (Gentest), 1 mM reduced NADP (NADPH)-generating system (NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate), and 10 mM MgCl₂. Reactions were initiated by the addition of midazolam and incubated in a shaking water bath at 37°C for 90 min (lung microsomes) and 30 min (liver microsomes). All reactions were terminated by the addition of an equal volume of ice-cold methanol, vortexed, and placed overnight at -20° C. Samples were centrifuged at 10,000 g at 4°C for 20 min to remove precipitated protein. One hundred microliters of the supernatant were used for HPLC analysis of midazolam metabolite formation. Samples were injected onto a Luna 100 \times 4.6-mm, 3- μ m column (Phenomenex, Torrance, CA). Metabolites were separated with a 26-min isocratic method [flow: 1.0 ml/min, mobile phase: 52% organic (37.5% methanol, 22.5% acetonitrile)-48% 10 mM potassium phosphate buffer] and monitored at an ultraviolet absorbance of 220 nm. Standard curves with known 1-OH MDZ and 4-OH MDZ metabolite concentrations were used. The amount of CYP3A activity was calculated for each replication and graphed to represent mean (SD) activity per picomole per milligram of protein per minute. All HPLC runs included the appropriate reaction blanks. Initial studies were performed to determine optimal protein concentration, substrate concentration, and incubation time, and all incubations were conducted in the linear range. Midazolam and metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) to verify their purity, and the metabolites generated from liver incubations were also analyzed by LC-MS.

Statistics. Data are reported as means (SD). Differences between 7-day-old and adult rats were determined by *t*-test, and significance was based on P < 0.05 (SigmaStat Software, Jandel Scientific, San Rafael, CA).

RESULTS

CYP3A1 quantitative mRNA and protein expression. Baseline expression of CYP3A1 mRNA in proximal airway epithelium of 7-day-old rats was similar to the expression found in adult rats. Likewise, CYP3A1 mRNA expression in 7-day-old rat pulmonary vein (cardiac and smooth muscle) was approximately equal to adult rat CYP3A1 mRNA expression (Fig. 1). Proximal airway and pulmonary vein relative expression values for the internal reference gene (GAPDH) were also not significantly different between age groups. In the proximal airway, the raw relative expression value for GAPDH was 29.66 (SD 0.76) in 7-day-old rats and 29.53 (SD 1.28) in adult rats. In the pulmonary vein, the relative expression value for GAPDH was 28.99 (SD 0.68) in 7-day-old rats and 29.96 (SD 1.96) in adult rats. In contrast to the similar mRNA expression, quantitative protein levels of CYP3A1 were greater in adult rats. Adult rat CYP3A1 immunoreactive protein levels in



Fig. 1. Steady-state site-specific cytochrome *P*-450 (CYP)3A1 RNA expression in proximal airway epithelium and pulmonary vein (cardiac and smooth muscle) from 7-day-old rats and adult rats. CYP3A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA expression was standardized to the steady-state RNA expression of GAPDH from adult rat isolated airway. GAPDH RNA expression did not vary between 7-day-old and adult rats. The amount of CYP3A1 RNA is a representation of 5 independent adult and 7-day-old rat samples (n = 5). There was no significant difference in 7-day-old and adult rat CYP3A1 RNA expression at P < 0.05.

Fig. 2. Western blots showing immunoreactive CYP3A1 protein expression. A: steady-state immunoreactive CYP3A1 protein expression from isolated airways of 4 adult rats (1-4) and 5 concentrations of adult rat liver protein loaded as an internal standard. B: steady-state immunoreactive CYP3A1 protein expression from isolated airways of 4 7-day-old rats (1-4)and 5 concentrations of adult rat liver protein loaded as an internal standard. C: relative abundance of pulmonary CYP3A protein expression calculated from internal protein standards. The amount of CYP3A1 protein is a representation of 4 independent adult and 7-day-old rat samples (n = 4). D: linear regression analysis of quantified adult rat liver protein. *Significantly different from adult at P < 0.05.



microdissected airways were 1.39-fold higher than 7-day-old rat CYP3A1 immunoreactive protein levels (P = 0.003; Fig. 2).

Distribution of immunoreactive CYP450 protein. Immunoreactive CYP3A1 protein was detected in both nonciliated (Clara) cells and ciliated cells in the proximal airway epithelium of 7-day-old and adult rats. Site-specific labeling of immunoreactive CYP3A1 protein in 7-day-old rats was located throughout the cytoplasm and in the apex of ciliated and nonciliated cells (Fig. 3A), whereas in adult rats site-specific labeling of immunoreactive CYP3A1 was detected in the apices of ciliated and nonciliated cells (Fig. 3B). In the distal airways of 7-day-old and adult rats, immunoreactive CYP3A1 was detected in nonciliated cells and there was very faint labeling of ciliated cells (Fig. 3, C and D). In the proximal and distal airway epithelium, adult rats had a higher number of cells labeled positive for CYP3A1 protein, and these cells also had a stronger signal compared with 7-day-old rats. However, in both age groups, a larger number of positive cells were detected in the proximal airway epithelium compared with distal airway epithelium.

For both age groups, CYP3A1 immunoreactive protein was detected, with a lower concentration of antibody in nonciliated cells in proximal airway epithelium than in ciliated cells, indicating higher levels of immunoreactive protein in nonciliated cells. CYP3A1 immunoreactive protein was detected in the apex of nonciliated cells at a dilution of 1:2,000 in adult rats and at a dilution of 1:1,000 in 7-day-old rats. However, for both age groups, at these respective dilutions, only a few positive ciliated cells were detected. A strong immunoreactive protein signal was seen in ciliated cells at a dilution of 1:1,500 in adult rats and at a dilution of 1:750 in 7-day-old rats. A similar pattern was present in the distal airway epithelium for both age groups but at higher antibody concentration.

In addition to airway epithelial cells, cardiac muscle cells surrounding the proximal pulmonary vein were heavily labeled

for immunoreactive CYP3A1 protein in both 7-day-old and adult rats (Fig. 3, E and F). We analyzed the cardiac muscle to determine whether it could potentially contribute to toxicity of known environmental cytotoxicants such as 1-nitronaphthalene. Unlike other known pulmonary CYP450s, which have not been shown in the cardiac muscle of pulmonary veins, CYP3A1 was heavily expressed in both 7-day-old and adult rats (27). CYP3A1 protein was detected in cardiac cells in adult rats at a dilution of 1:10,000 and in 7-day old rats at 1:1,500, and the signal intensity increased with an increase in antibody concentration. The characteristic cardiac muscle cell striations are visible in an immunohistochemistry image of 7-day-old rat pulmonary vein (Fig. 3E) and in a toluidine blue-stained 0.5-µm-thick plastic section (Fig. 3G). Highly variable CYP3A1 immunoreactive protein labeling was observed in samples of adult rat pulmonary vein. There was no immunoreactive CYP3A1 protein labeling in the pulmonary arteries and no visible labeling in the pulmonary veins located in the distal airways of both 7-day-old and adult rats (data not shown).

Microsomal activity of CYP3A1. CYP3A1 activity was detected in microsomal protein from isolated airways of both 7-day-old and adult rats. In contrast to the mRNA expression, there was a statistically significant difference in midazolam metabolism rates. CYP3A1 isozyme activity in the lung of adult rats was statistically higher than in 7-day-old rats for the generation of 4-OH MDZ metabolite ($P \le 0.03$), and there was not a statistical difference in the generation of the 1-OH MDZ metabolite (P = 0.347). In 7-day-old rats, 4-OH MDZ and 1-OH MDZ were generated at a rate of 1.44 (SD 0.078) and 0.421 (SD 0.204) pmol·mg protein⁻¹·min⁻¹, respectively (Fig. 4). In adult rats, 4-OH MDZ and 1-OH MDZ were generated at a rate of 1.867 (SD 0.273) and 0.578 (SD 0.192) pmol·mg protein⁻¹·min⁻¹, respectively (Fig. 4).











Fig. 3. Comparison of immunohistochemical localization of CYP3A1 immunoreactive protein in nonciliated (NC) and ciliated (Cil) cells of the proximal airway epithelium of 7-day-old rats (A) and adult rats (B) and the distal bronchiolar epithelium of 7-day-old rats (C) and adult rats (D) with Nomarski differential interference contrast at $\times 60$. E and F: comparison of CYP3A1 immunoreactive protein localized in the proximal pulmonary vein (smooth and cardiac muscle) of 7-day-old rats (E) and adult rats (F) at \times 40. G: cardiac muscle surrounding the proximal pulmonary vein of a 7-day-old rat (0.5-µmthick section) at $\times 40$. *H*: negative control for the proximal airway at ×40. Magnification bar represents 5 µm. The distribution of CYP3A1 immunoreactive protein is a representation of 5 independent adult and 7-day-old rat samples (n = 5).

H H

CYP3A activity was also detected in microsomal protein from livers in 7-day-old and adult rats. CYP3A activity rates in the liver of adult rats were statistically higher than in 7-day-old rats for the generation of 1-OH MDZ metabolite ($P \le 0.016$), and there was not a statistical difference in the generation of the 4-OH MDZ metabolite ($P \le 0.076$). In 7-day-old rats, 4-OH MDZ and 1-OH MDZ were generated at a rate of 301.51 (SD 65.81) and 78.26 (SD 25.51) pmol·mg protein⁻¹·min⁻¹, respectively (Fig. 5). In adult rats, 4-OH MDZ and 1-OH MDZ were generated at a rate of 420.99 (SD 129.22) and 150.24 (SD 50.47) pmol·mg protein⁻¹·min⁻¹, respectively (Fig. 5). Because it is essential to understand the balance between first-pass metabolism and the resulting toxicity, we measured CYP3A1 activity in the liver.

DISCUSSION

Given that disparities in pulmonary metabolism of xenobiotics occur between postnatal and adult animals (12, 50, 59) it is critical to understand levels of CYP450 isozymes at different stages of lung development when evaluating chemical exposure limits (20, 21). The lungs of postnatal rats are continuing to grow, and differentiation of pulmonary epithelial cells differs by airway generation both temporally and spatially (45, 48, 62). Expression of CYP450 isozymes that are thought to be relevant for bioactivation of xenobiotics increases with the differentiation of epithelial cells. Furthermore, from previous isozymes studied, fully differentiated expression is species specific and generally does not occur until late postnatal age. In this present study, we found that there are age-related differences in the type of CYP3A1 expression. Ciliated and nonciliated cells in the proximal airway of postnatal and adult rats both have immunoreactive CYP3A1 protein. Whereas expression of CYP3A1 mRNA in differentiating airway cells of postnatal rats is similar to that in fully differentiated cells of adults, immunoreactive protein levels and enzymatic activity are 28% and 23% lower, respectively, in postnatal airways compared with adult airways.

A unique aspect of this study is the definition of gene expression in cell populations from individual airway generations based on the extraction of mRNA from cells isolated by



Fig. 4. Pulmonary CYP3A1 activity levels demonstrated by the rate of midazolam metabolism in 7-day-old and adult rat isolated airway microsomal protein from pooled microdissected airways. The amount of CYP3A1 activity for 7-day-old rats was calculated from 4 independent pools of microsomal protein from microdissected airways (10–20 rats per pool, n = 4). The amount of CYP3A1 activity for adult rats was calculated from 3 independent pools of microsomal protein from microdissected airways (10–12 rats per pool, n = 3). Data represent means (SD). 1-OH MDZ, 1-hydroxymidazolam; 4-OH MDZ, 4-hydroxymidazolam. Differences between 7-day-old and adult rats were determined by *t*-test. *Significantly different from adult at P < 0.05.

laser capture microdissection. In this case, the target cell population is those cells that contain CYP450 mRNA from the proximal airway epithelium. Isolating generation-specific airway cell epithelium is important because the lung contains over 40 different cell types and only 4 or 5 of these cells have CYP450 mRNA expression. We found that CYP3A1 mRNA expression in airway epithelium is similar between 7-day-old and adult rats, matching the developmental expression of pulmonary mRNA reductase in rats (32). In contrast, the developmental expression of mRNA for other isozymes (CYP1A1 and CYP2B1) has a longer postnatal period of maturation in the rat (17, 32). However, the findings for CYP1A1 and CYP2B1 mRNA expression were based on isolating mRNA from whole lung tissue, which dilutes the CYP-specific mRNA in each sample. In contrast, mRNA isolation by laser capture microdissection enriches the CYPspecific mRNA. This is the first study to isolate CYP450 mRNA by laser capture microdissection from the lung, and the technique may result in findings different from those when mRNA is isolated from homogenates of the whole lung.

Even though postnatal and adult rats have similar expression of CYP3A1 mRNA, the mRNA must be transcribed and translated into a functional protein. We found that the level of pulmonary CYP3A1 immunoreactive protein is 28% lower in postnatal rats than in adults. These data are consistent with previously reported postnatal immunoreactive protein levels for other CYP isozymes including reductase, 4B, and 2B in rabbit (49), 2B and 2F2 in mice (14), 1A1 and 1B1 in goat (10), and 2B and reductase in rat (23). Plopper et al. (49) correlated lower detectable immunoreactive protein levels in the rabbit to CYP450s in undifferentiated nonciliated cells, and our data suggest that a similar event may occur with the CYP3A1 isozyme in rats. In the proximal and distal airway epithelium, adult rats had a stronger signal and higher number of CYP3A1positive labeled nonciliated and ciliated cells compared with 7-day-old rats. In postnatal rats, both nonciliated and ciliated cells are undifferentiated and differentiation of these cells occurs in a proximal-to-distal manner (24, 45, 48, 62). The total cell volume of nonciliated bronchiolar epithelial cells in 7-day-old rats only contains 3-7% smooth endoplasmic reticulum and contains low levels of CYP450 protein. By adulthood, there is an increase in total CYP450 protein and the total volume of smooth endoplasmic reticulum substantially increases to $\sim 25\%$ of the total cell volume (23). The lower levels of smooth endoplasmic reticulum and high cytoplasmic glycogen content in postnatal rat bronchiole epithelial cells may account for the difference detected in the location of intracellular CYP3A1 immunoreactive protein between postnatal and adult rats. The discordance between mRNA expression and protein levels observed in postnatal rats may be due to several factors, including efficiency of mRNA transcription and/or translation, stability of message, posttranscriptional regulation, posttranslational regulation, and protein degradation. Regulation of these factors may differ between postnatal and adult rats and could explain why there was no discordance between mRNA and protein expression in adult rats. Another factor could be differences in the specificity of methods used to isolated mRNA and protein. Although the mRNA expression is generation specific, because of limitations of current protein methods the quantitative protein expression represents protein expression from microdissected airways. However, the density and distribution of immunoreactive protein in immunohistochemical sections in the airways match protein levels from Western blots.

Because postnatal rats have lower pulmonary CYP3A1 protein levels compared with adults, we investigated whether this translates into lower CYP3A1 enzymatic activity in postnatal rats than adults. When evaluating specific CYP450 enzyme activity it is important to note the specificity of the substrates



Fig. 5. Liver CYP3A1 activity levels demonstrated by the rate of midazolam metabolism in 7-day-old and adult rat microsomal protein. The amount of CYP3A1 activity for 7-day-old rats was calculated from 5 independent pools of liver microsomal protein (10–20 rats per pool, n = 5). The amount of CYP3A1 activity for adult rats was calculated from 5 independent pools of liver microsomal protein (10–14 rats per pool, n = 5). Data represent means (SD). Differences between 7-day-old and adult rats were determined by *t*-test. *Significantly different from adult at P < 0.05.

used. p-Nitrophenol, coumarin, phenacetin, 7-benzyloxyresorufin, and chlorzoxazone are all substrates for the CYP3A isozyme; however, they are also substrates for CYP1A, CYP2B, CYP2C, and CYP2E (28, 70). For our studies, we determined the specific catalytic ability of CYP3A1 in isolated airway microsomal protein with a specific substrate, midazolam. The biotransformation of midazolam by CYP3A has been well characterized in both humans and rats (7, 19, 28, 29, 66). Although midazolam is also metabolized by CYP2C11 (28), we found no evidence of CYP2C11 immunoreactive protein in the lung of either postnatal or adult rats (unpublished data). In concordance with CYP450 immunoreactive protein, isolated airway microsomes from postnatal rats generated midazolam metabolites at a rate that was 23% lower than in adults. This is consistent with previously reported postnatal steady-state pulmonary enzymatic activity for other CYP isozymes, including 4B and 2B in the rabbit (49), 2B and 1A1 in the goat (9), 2B and 2F2 in the mouse (14), and 1A1, NADPH-reductase, and 2B in the rat (18, 23). The lower levels of CYP3A1 enzymatic activity in 7-day-old rats compared with adult rats indicates age-dependent maturation of the CYP3A1 isozyme. A similar pattern has been reported for the CYP2B isozyme, where enzymatic activity is lower in 7-day-old rats and does not reach adult levels until 50 days of age (23).

Similar to humans (1), CYP3A1 immunoreactive protein was detected in ciliated cells in postnatal and adult rat intrapulmonary airways (generations 2 and 3) and was also found in nonciliated cells. Ciliated cells are the predominant cell type in the intrapulmonary airways and are usually considered to contain little, if any, CYP450 protein; immunoreactive protein for the CYPP450 isozymes 1A1, 2B1, 2F2, and 2E1 is not detected (23, 25, 33, 42, 67). However, studies have reported ciliated cell toxicity from CYP450-activated toxicants such as 1-nitronaphthalene (13, 26, 41, 52, 55, 56). The mechanism of ciliated cell toxicity is still unclear. One proposed mechanism is diffusion of reactive intermediates from Clara cells to neighboring ciliated cells.

It still remains unclear why undifferentiated and developing cells of postnatal animals that have lower detectable levels of pulmonary CYP450s have a heightened susceptibility to CYP450-activated toxicants naphthalene (12) and 4-ipomeanol (50, 59) at the same or lower doses. It was recently reported that postnatal rats are more susceptible than adults to pulmonary injury from another CYP450-activated toxicant, 1-nitronaphthalene, which targets both ciliated and nonciliated cells (13). 1-Nitronaphthalene may also be a substrate for CYP3A1 acting in a similar manner to numerous known compounds in rats and humans that are metabolized by the CYP3A isozyme and also act as a substrate, including erythromycin, dexamethasone, SKF-525A, and phenobarbital (8, 34, 37, 39). The localization of CYP3A1 protein in ciliated cells may be a key factor in 1-nitronaphthalene pulmonary toxicity. Another explanation for increased susceptibility of postnatal animals is a discrepancy between activation and detoxification enzymes. An inadequate ability to detoxify the reactive intermediates generated by these toxicants may prevent complete removal of reactive metabolites, allowing metabolites to bind to critical macromolecules.

In summary, the present study has shown that postnatal rats have detectable levels of pulmonary CYP3A1. Furthermore, CYP3A1 mRNA expression appears not to be age dependent, whereas data indicate an age-dependent pattern for CYP3A1 protein levels and enzyme activity. In adult animals, research has demonstrated that a close relationship exists between increased lung toxicity and high levels of cytochrome monooxygenases, abundant smooth endoplasmic reticulum, presence of CYP450 genes, and generation of reactive metabolites (5, 33, 58, 61). However, research has also shown that postnatal animals are more susceptible than adults to lung toxicity from some environmental toxicants even though they have lower levels of cytochrome monooxygenases, less smooth endoplasmic reticulum, and less enzymatic activity (6, 12, 14, 18, 23, 24, 49, 50). Because the lung is a primary target for air pollutants and CYP450s metabolize many of these compounds, it is important to consider the developmental phase of CYP450s in the lung when evaluating and establishing environmental exposure limits for children.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the guidance and technical expertise of Dr. Alan Buckpitt and Dr. Marion Miller and the editorial expertise of Dr. Suzette Smiley-Jewell.

GRANTS

The University of California, Davis is a National Institute of Environmental Health Sciences (NIEHS) Center for Environmental Health Sciences (ES-05707), and support for core facilities used in this work is gratefully acknowl-edged. This research has been supported in part by NIEHS Grants ES-06700 and ES-0411 and National Heart, Lung, and Blood Institute Training Grant HL-007013 as well as a grant from the US Environmental Protection Agency's (EPA) Science to Achieve Results (STAR) Program (R827442). Although the research described in the article has been funded in part by the EPA's STAR program (Grant R827442), it has not been subjected to any EPA review and therefore does not necessarily reflect the views of the Agency.

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