Lung cancer is increasingly common in women (Patel et al., 2004). Although smoking incidence clearly has contributed to the rise of lung cancer in women, what is also clear is that lung cancer is a different disease in women. Both smoking and nonsmoking women have a greater incidence of adenocarcinoma than their respective smoking and nonsmoking male counterparts (Bain et al., 2004; Nordquist et al., 2004). This observation has led to the hypothesis that circulating hormones, in particular, estrogens, influence pulmonary susceptibility, especially in women exposed to tobacco smoke (Stabile and Siegfried, 2004). In support of this argument, several studies have found that human lung tumors express estrogen receptors and have a proliferative response to estrogen (Stabile et al., 2002). Furthermore, lung tumors have a higher frequency of both estrogen receptor types than nontumor lung tissue in women (Fasco et al., 2002). Although sex differences in pulmonary epithelial responses are clear after respiratory disease develops, the factors that result in increased susceptibility by sex remain largely unknown. One factor that may influence susceptibility is the extent and pattern of acute injury by airway region. This, in turn, can be influenced by formation of toxic metabolites by cytochrome P450 monoxygenases. These may be regulated by gonadal hormones. However, hormonal regulation of P450s in the lung has received little attention.

As a tool for probing sex differences in metabolic activation in the lung, we have elected to study the simplest polycyclic aromatic hydrocarbon, naphthalene. Humans are exposed to naphthalene from many sources. It is the most abundant polycyclic aromatic hydrocarbon found in sidestream smoke, is present in automobile emissions, and is the primary polyaromatic pollutant present in ambient air (International Agency for Research on Cancer, 2002). Humans are exposed to naphthalene, regardless of route, at sufficient levels so that it circulates within the body; it has been found in adipose tissue and breast milk (Pelizzari et al., 1982; Stanley, 1986). Susceptibility of mice to naphthalene correlates with formation of the toxic metabolite by cytochrome P450 isozyme 2F2 (CYP2F2). Mice have an abundance of CYP2F2, particularly in Clara cells in the distal bronchioles (Buckpitt et al., 1995).

Chronic naphthalene vapor exposure resulted in an increased incidence of pulmonary alveolar/bronchiolar carcinomas, but only in female mice (NTP, 1992). Sex differences in response were also observed after i.p. administration of naphthalene. Female mice (random, undetermined estrous cycle stage) have an earlier onset as well as a different pattern and extent of acute inflammatory injury compared with males at the same dose (Van Winkle et al., 2002). Furthermore, pulmonary subcompartments from female mice metabolized naphthalene more rapidly, with a greater proportion of dihydrodiol metabolite formation than males. However, there was considerable variability in the gene expression for CYP2F2, a major P450.
that metabolizes naphthalene in the mouse lung. One hypothesis for the increased variability in females was that it was due to the influence of hormonal changes during estrous cycling. Accordingly, this study was designed to address the following question: do hormonal changes associated with the estrous cycle alter naphthalene metabolism in the lung? In this study we describe the effects of normal hormonal variation on metabolic activation of naphthalene in the extrapulmonary (trachea and bronchi) and intrapulmonary (bronchi to terminal bronchioles) airways of female mice. Substantial variations in bioactivation of naphthalene by stage of estrous cycle support the conclusion that hormonal influences regulate metabolism of naphthalene in the mouse lung. This is the first study to show that naphthalene metabolism is affected by estrous cycle stage.

Materials and Methods

Animals and Tissue Preparation. All animals were adult (>8 weeks old) NIH:Swiss-Webster male and virgin female mice (Harlan, Indianapolis, IN). For experiments with cycle-staged female mice, female mice were housed separately from males and were induced to cycle using exposure to male mouse urine-soaked bedding (Whitten 1966; Dalal et al., 2001). All mice were housed on a 12-h light/dark cycle and all vaginal swabs and necropsies were performed in the first 3 h of the light cycle. Cytology on vaginal swabs was used to define cycle stage. Vaginal swabs were taken on day 1 after the 3-week acclimatization period. Male bedding was added to female mouse cages on this day. Noncycling female mice were not exposed to male mouse urine-soaked bedding. Vaginal swabs were taken 3 days later (day 4). Criteria were as described by Dalal et al. (2001) and stage assignment was based on classification of cells and relative abundance of each cell type (percentage of parabasal, intermediate, cornified, neutrophils). Only female mice that fell into one of three groups (noncycling, proestrous, estrus) based on sequential vaginal cytology were used. Mice were necropsied immediately after classification. The noncycling group was classified as in diestrus (>50% neutrophils) on day 1 and remaining in diestrus through day 4, via cytology. The proestrus group was defined as in proestrus (>25% parabasal epithelial cells, <10% neutrophils) via vaginal cytology. The estrus group was classified as in estrus (>50% superficial epithelial cells, 0 neutrophils) via cytology. Estradiol levels for each of the groups were determined using a commercially available ELISA (IBL, Hamburg Germany) on serum obtained at necropsy.

Naphthalene Metabolism. Cytochrome P450-dependent naphthalene metabolism was measured in two airway regions: extrapulmonary airways and intrapulmonary airways from the left lobe using methods that have been described in detail elsewhere (Plopper et al., 1991; Van Winkle et al., 2002). To define the extent of naphthalene metabolism in female compared with male mice, live intact microdissected airways were obtained from a minimum of five male and five female mice of each stage. The explanted airways were incubated with naphthalene in vitro and the metabolites from this reaction were trapped with glutathione (GSH). Incubations contained GSH (1 mM), glutathione transferase (5 units), and 0.5 mM substrate in a total volume of 0.5 ml in a sealed vial that was incubated at 37°C for 2 h. Total naphthalene metabolism was measured by high-performance liquid chromatography detection of the products 1,2-dihydroxy-1,2-dihydronaphthalene (dihydrodiol) and glutathione conjugates (Fig. 1) from naphthalene oxide, as described previously and modified by Shultz et al. (1999).
Real-Time Reverse Transcription-Polymerase Chain Reaction. Quantification of CYP2F2 gene expression was performed on intrapulmonary and extrapulmonary airways isolated from lungs inflated with RNaLater (Ambion, Austin, TX) as described in Baker et al. (2004). Extrapulmonary airway samples included trachea and lobar bronchi. All RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA). RNA (200 ng) was reverse-transcribed in a 25-ml reaction mixture containing 1X TaqMan RT buffer, 5.5 mM MgCl₂, 500 ml of deoxyribonucleoside-5'-triphosphates, 2.5 ml of random hexamers, 0.4 U/ml ribonuclease inhibitor, and 1.25 U/ml multiscrbe reverse transcriptase. The reaction mixtures were incubated at 25°C for 10 min, 37°C for 60 min and at 95°C for 5 min. Primer and probe sequences were designed using Primer Express software (Applied Biosystems). Specificity of primer probe sets was determined by agarose gel electrophoresis of PCR reaction products revealing a single band of expected size. Individual PCRs contained 1X TaqMan Mastermix, 1.25 ml of cDNA, 900 nM 5' and 3' primers, and 100 nM probe. PCRs were performed with an ABI 5700 sequence detection system using the following cycling protocol: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Results were calculated using the comparative Ct method. Briefly, the threshold cycle, Ct, is defined as the point at which the first significant increase in fluorescence is observed. For each sample, a dCt value is obtained: the difference between the CYP2F2 and the internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPD), (CtCYP2F2 − CtgAPD). A calibrator value was obtained by calculating the average of the male extrapulmonary group dCt values. The dCt values of each sample were obtained by subtracting the dCt(calibrator value) from the dCt(calibrator value) + dCt(sample). Results are expressed as a fold difference in gene expression relative to the male extrapulmonary group RNA and was calculated using the formula: fold change = 2^(ddCt). Results were analyzed using the Kruskal-Wallis test and Dunn’s multiple comparison post hoc test. P values less than 0.05 were considered significant.

Statistics. Results were analyzed using analysis of variance and Scheffe’s post hoc test. P values less than 0.05 were considered significant. All values are means ± 1 standard deviation with a minimum sample size of five animals per group.

Results

Adult female mice were manipulated into semisynchronous cycling. Cycling was confirmed using a series of vaginal swabs and cytology (see examples in Fig. 2A). Serum estradiol was measured by ELISA and was found to vary significantly by cycle stage, with female mice in proestrus having significantly less circulating estradiol than females at other cycle stages (Fig. 2B).

At specific cycle times, extrapulmonary (trachea and bronchi, Fig. 3) and intrapulmonary (bronchioles, Fig. 4) airways were microdissected and incubated with naphthalene. We measured the products of naphthalene metabolism (Fig. 1) in female mice that were estradiol-high (estrus stage of cycle) and estradiol-low (proestrus stage of cycle) using high-performance liquid chromatography (Figs. 3 and 4). Total naphthalene metabolism in the extrapulmonary airways was significantly less in noncycling female mice compared with females in proestrus (Fig. 3A). In the extrapulmonary airways, the formation of glutathione conjugate 2 as a percentage of total metabolism was the least in the female mice in estrus, and this was significantly different from mice in proestrus and in males (Fig. 3B). Conversely, female mice in estrus formed significantly more dihydrodiol as a percentage of total extrapulmonary airway metabolism than females in proestrus and noncycling females (Fig. 3C). Dihydrodiol formation was not significantly different for females in estrus versus males (P = 0.11).

In the intrapulmonary airways, total naphthalene metabolism was significantly greater (P < 0.05) in mice at the proestrus stage of the estrous cycle compared with females in estrus, noncycling females, or males (Fig. 4A). Formation of naphthalene glutathione conjugate 2 (derived from the 1α,2β-epoxide), expressed as a percentage of total metabolism (Fig. 4B), was also significantly greater in the intrapulmonary airways of proestrus females compared with noncycling females or males in estrus. Compared with males, only the estrus and noncycling groups were significantly different in the percentage of metabolism that was conjugate 2 (Fig. 4B). Dihydrodiol was the greatest percentage of total naphthalene metabolism in the estrus group, and this was significantly different from all other groups (Fig. 4C).

CYP2F2 gene expression was measured using real time RT-PCR in microdissected extra- and intrapulmonary airways (Fig. 5). In the extrapulmonary airways, the noncycling females had significantly less CYP2F2 gene expression than proestrus or estrus animals, but were not different from males. In the intrapulmonary airways, all female groups had significantly higher CYP2F2 expression compared with males, but the female groups did not differ from each other.

Discussion

Site-specific metabolism is an important determinant of toxicity in many organs, but especially in the lung. The focal nature of lung toxicity by metabolically activated toxicants is partly due to the fact that lung cell biology varies greatly by location within the lung and by cell type (Plopper, 1993; Plopper et al., 2001). Lung cells with metabolic capability include Clara cells, alveolar type 2 cells, and macrophages, and these cells are located in distinct subcompartiments.

FIG. 2. A, examples of vaginal cytology found in the four estrous cycle stages. Mice in proestrus, estrus, and noncycling (diestrus) were used for this study and were easily sorted into groups based on the relative abundance of cornified, basal, and inflammatory cells found in swabs. B, serum levels of estradiol measured by ELISA in the serum of male and female mice. Estradiol levels differed significantly between proestrus and estrus cycle stages.
within the lung. Clara cells, the cells that contain the greatest amount of pulmonary P450s, are restricted to the conducting airways. For some compounds, injury may be found preferentially in the most distal airways where Clara cells occur in the greatest abundance. Injury may also be influenced by toxicant distribution in the lung, particularly if inhaled (Postlethwait et al., 2000). The conducting airways comprise less than 5% of the total lung volume. For this reason, we chose to study sex differences in pulmonary metabolism of...
naphthalene using in vitro incubations of the conducting airway fraction of the lung obtained by microdissection (Plopper et al., 1991). Airways were subdivided into two broad and easily distinguishable classes: extrapulmonary (bronchi and trachea) and intrapulmonary (bronchioles). As expected total naphthalene metabolism was greatest in the more distal, intrapulmonary airways (compare Fig. 3 with Fig. 4) (Buckpitt et al., 1995). However, the pattern of naphthalene metabolism was also different by airway generation, with differences in total metabolism most marked in the intrapulmonary airways of all groups compared with females in proestrus. The highest rates of formation of naphthalene metabolites were found in the intrapulmonary airways of female mice in the proestrus stage. The lung has hormone receptors for both estrogen and progesterone (Couse et al., 1997; Fascio et al., 2002; Gonzalez-Arenas et al., 2003). However, little is known about the effect of circulating hormones or their nuclear hormone receptors on metabolism of xenobiotics in the lung. The results for the intrapulmonary airways in the current study are within the range of naphthalene metabolism reported previously for subdivisions of the intrapulmonary bronchioles (minor daughters and terminal bronchioles) in male and random cycling female mice (Van Winkle et al., 2002). Naphthalene metabolism was greatest in the intrapulmonary airways when estrogen levels were lowest (proestrus). This was also true for the extrapulmonary airways; however, there was not a difference between the estradiol high (estrus) and estradiol low groups, thereby suggesting that another hormonal influence besides estrogen likely has a role in the trachea and bronchi. Metabolism of naphthalene to the dihydrodiol was greatest as a percentage of total metabolism in the estradiol low condition in both intran- and extrapulmonary airway segments. It is possible that micr-osal epoxide hydrolase may also be regulated by estrogen, again, perhaps in the context of other hormones and their receptors. Previous studies of hepatic microsomal epoxide hydrolase activities in intact and gonadectomized male and female mouse liver found reduction in activity in the castrated animals (Inoue et al., 1993). In the rat lung, progesterone and estrogen receptors have been found to vary with the estrous cycle, with both progesterone receptor isoforms highest during proestrus and lowest during estrus (Gonzalez-Arenas et al., 2004).

Figs. 5. CYP2F2 gene expression in the extrapulmonary and intrapulmonary airways measured by real-time RT-PCR and expressed as fold change relative to male extrapulmonary airways. ∗, significantly different from proestrus group; ‡, significantly different from estrus group; †, significantly different from estrous group. Significance accepted at $P < 0.05$. is important to point out that steroid receptor or cofactor expression may influence naphthalene metabolism. If mice have a pattern of receptor expression similar to that which has been reported for rat lung, then naphthalene metabolism may potentially be influenced by progesterone receptor expression and its effects on the enzymes involved either directly or through cofactor expression.

Hormonal regulation of P450 is well recognized in reproductive organs and the liver. Estrous cycle regulates the metabolism of dimethylbenz[a]anthracene in rat liver microsomes (Fu et al., 2003). Comparison of hepatic P450 activity in female rats at different stages of estrus with males found higher total P450 content, aniline hydroxylase (CYP2E1), ethoxycoumarin O-deethylase (CYP2A), and aminopyrine N-demethylase activities in males than in females at any stage (Watanabe et al., 1997). Total liver microsomal P450 content has also been found to be higher in male mice than in female mice (Guo et al., 1993). Hepatic cytochrome c reductase is significantly lower at proestrus or diestrus compared with estrus in female rats (Watanabe et al., 1997). However, NADPH:cytochrome c reductase activity in proestrus females was similar to that in males and was higher than that found in females in estrus or diestras stages. Our study suggests possible hormonal regulation of the activity of enzymes involved in naphthalene bioactivation in the mouse lung. However, gene expression data suggest that regulation of CYP2F2 is only part of the explanation for altered naphthalene metabolism by cycle stage; CYP2F2 expression did not vary significantly by estrous cycle stage in intrapulmonary airways. In contrast CYP2F2 expression did vary significantly by cycle stage in the extrapulmonary airways, suggesting that different processes regulate CYP2F2 gene expression in intra- versus extrapulmonary airways. Furthermore, female mice expressed significantly more CYP2F2 in their intrapulmonary airways than did male mice, correlating with the increased cytotoxicity of female mice to naphthalene reported previously (Van Winkle et al., 2002).

We conclude that naphthalene metabolism is influenced by the estrous cycle in the lungs of female mice and varies by airway level. Furthermore, the pattern of changes suggests that naphthalene conjugate formation may be inhibited by estradiol, but dihydrodiol formation in proximal airways is influenced by other hormones. Our study suggests that hormonal regulation of enzymes (such as P450s or epoxide hydrolase), or their cofactors, in the lung of females influences metabolism of naphthalene. Formation of increased levels of toxic metabolites may, in turn, influence the extent of toxicity or tumor formation, issues that may underlie the differential susceptibility of females to lung cancer.

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References

