DMD Fast Forward. Published on August 5, 2005 as doi:10.1124/dmd.105.005124

ESTROUS CYCLE ALTERS NAPHTHALENE METABOLISM IN FEMALE MOUSE AIRWAYS

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Running Title: Estrous cycle alters naphthalene metabolism in airways

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Text pages: 10.5
Tables: 0
Figures: 5
References: 27
Words in ABSTRACT: 230
Words in INTRODUCTION: 580
Words in DISCUSSION: 932

Non standard abbreviations: GSH Tx = Glutathione Transferase

ABSTRACT

Previous studies have shown variability in naphthalene cytotoxicity, expression of CYP2F2 gene and protein and naphthalene metabolism in random cycling female mice (NIH:Swiss). CYP2F2 metabolizes naphthalene to cytotoxic metabolites in lungs of mice. This study was designed to address the question: Do hormonal changes associated with the estrus cycle alter metabolism of naphthalene in the lung? Adult virgin female mice were manipulated into defined stages of the reproductive cycle: estrus, proestrus and noncycling. Cycling was confirmed by cytology on vaginal swabs. At specific cycle times, extrapulmonary (trachea and bronchi) and intrapulmonary (bronchiolar) conducting airways were microdissected from the lung parenchyma, incubated with naphthalene and the products of naphthalene metabolism were trapped and measured using HPLC. Circulating estradiol levels were measured at necropsy using ELISA. CYP2F2 gene expression was determined by airway level using real time RT PCR and did not vary by estrous cycle stage in intrapulmonary airways but did in extrapulmonary airways. Metabolism of naphthalene varied significantly by estrous cycle stage with the highest level of total metabolism occurring in proestrus (when estrogen is lowest) in intrapulmonary airways. Total activity and metabolite profiles in both extrapulmonary and intrapulmonary airways were affected by cycle stage. We conclude that the hormonal patterns associated with different stages of the estrous cycle 1) alter metabolism of naphthalene in the lungs of mice and 2) alter naphthalene metabolism differentially in extrapulmonary vs intrapulmonary airways.

INTRODUCTION

Lung cancer is increasingly common in women (Patel et al. 2004). While 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 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). Further, lung tumors have a higher frequency of both estrogen receptor types than nontumor lung tissue in women (Fasco et al. 2002). While 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 monooxygenases. 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 hydrocarbons, 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 (Pellizzari 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 airway epithelial injury compared to males at the same dose (Van Winkle et al. 2002). Further, 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 estrus 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 estrus cycle supports 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.

DMD Fast Forward. Published on August 5, 2005 as DOI: 10.1124/dmd.105.005124
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METHODS:

Animals and Tissue Preparation: All animals were adult (>8 wks old) NIH Swiss Webster male and virgin female mice (Harlan Laboratories). 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 12/12 light/dark cycle and all vaginal swabs and necropsies were performed in the first 3 hours of the light cycle. Cytology on vaginal swabs was used to define cycle stage. Vaginal swabs were taken one day (Day 1) after the three 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 three days later (Day 4). Criteria were as described by Dalal and coworkers and stage assignment was based on classification of cells and relative abundance of each cell type (% of parabasal, intermediate, cornified, neutrophils). Only female mice that fell into one of three groups (noncycling, proestrus, 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. Proestrus group was defined as in proestrus (>25% parabasal epithelial cells, <10% neutrophils) via vaginal cytology. 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 P-450 dependent naphthalene metabolism was measured in two airway regions: extrapulmonary airways and intrapulmonary airways from the left lobe using methods which have been described in detail elsewhere (Plopper et al. 1991; Van Winkle et al. 2002). To obtain all the intrapulmonary airways, the lung is inflated to capacity with a low melting temperature agarose and airways were isolated under a high resolution dissecting microscope. These airways can be used for studies of region-specific metabolism and for tissue explants and contain intact fully functioning cells when they are incubated with naphthalane (Plopper et al. 1991; Van Winkle et al. 1996). To define the extent of naphthalene metabolism in female compared to male mice, live intact microdissected airways were obtained from a minimum of 5 male and 5 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 (1mM),

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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 hrs. Total naphthalene metabolism was measured by HPLC detection of the products 1, 2-dihydroxy-1,2-dihydronaphthalane (dihydrodiol) and glutathione conjugates (Figure 1) from naphthalene oxide, as described previously and modified by Shultz and coworkers (Shultz et al. 1999).

Real-time reverse transcription polymerase chain reaction: Quantitation of CYP2F2 gene expression was performed on intrapulmonary and extrapulmonary airways isolated from RNA later inflated lungs as described in (Baker et al. 2003). 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 $MgCl_2$, 500 ml dNTPs, 2.5 ml random hexamers, 0.4 U/ml ribonuclease inhibitor, 1.25 U/ml multiscribe reverse transcriptase. The reaction mixtures were incubated at 25°C for 10 min, 37°C for 60 min and 95°C for 5 min. Primer and probe sequences were designed using Primer Express software (Applied Biosystems, Foster City CA). Specificity of primer probe sets was determined by agarose gel electrophoresis of PCR reaction products revealing a single band of expected size. Individual PCR reactions contained 1X TaqMan Mastermix, 1.25 ml of cDNA, 900 nM 5' and 3' primers and 100 nM probe. PCR reactions were performed with an ABI 5700 sequence detection system using the following cycling protocol: 50°C for 2 min, 95° for 10 min and 40 cycles of 95°C for 15 s, 60°C 1 for 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, the difference between the CYP2F2 and the internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPD), ($Ct_{CYP2F2} - Ct_{GAPD}$). A calibrator value was obtained by calculating the average of the male extrapulmonary group dCt values. The ddCt values of each samples was obtained by subtracting the $dCt_{(experimental\ sample)} - dCt_{(calibrator\ value)}$. 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 Kruskall-Wallis test and Dunn's multiple comparison post hoc test. p values less than 0.05 were considered significant.

Statistics: Results were analyzed using ANOVA 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 5 animals per group.

RESULTS:

Adult female mice were manipulated into semi-synchronous cycling. Cycling was confirmed using a series of vaginal swabs and cytology (see examples Figure 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 (Figure 2B).

At specific cycle times, extrapulmonary (trachea and bronchi, Figure 3) and intrapulmonary (bronchioles, Figure 4) airways were microdissected and incubated with naphthalene. We measured the products of naphthalene metabolism (Figure 1) in female mice that were estradiol high (estrus stage of cycle) and estradiol low (proestrus stage of cycle) using HPLC (Figures 3 and 4). Total naphthalene metabolism in the extrapulmonary airways was significantly less in noncycling female mice compared to females in proestrus (Figure 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 (Figure 3B). Conversely, female mice in estrus formed significantly more dihyrodiol as a percentage of total extrapulmonary airway metabolism than females in proestrus and noncycling females (Figure 3C). Dihydrodiol formation was not significantly different for females in estrus vs 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 to females in estrus, to noncycling females or to males (Figure 4A). Formation of naphthalene glutathione conjugate 2 (derived from the 1R,2S-epoxide), expressed as a percentage of total metabolism (Figure 4B), was also significantly greater in the intrapulmonary airways of proestrus females compared to noncycling females or females in estrus. Compared to males, only the estrus and noncycling groups were significantly different in the percent of metabolism that was conjugate 2 (Figure 4B). Dihydrodiol was the greatest percentage of total naphthalene metabolism in the estrus group and this was significantly different from all other groups (Figure 4C).

CYP2F2 gene expression was measured using real time RT-PCR in microdissected extra and intrapulmonary airways (Figure 5). In the extrapulmonary airways, the noncycling females had significantly less CYP2F2 gene expression that proestrus or estrus animals, but were not different from males. In the

intrapulmonary airways, all female groups had significantly higher CYP2F2 expression compared to 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 subcompartments within the lung. Clara cells, the cell that contains 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 2 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 Figure 3 with Figure 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 to 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; Fasco 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 intra and extrapulmonary airway segments. It is possible that microsomal epoxide hydrolase may also be regulated by estrogen, again perhaps in 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). It is important to point out that steroid receptor or co-factor expression may influence naphthalene metabolism. If mice have a similar pattern of receptor expression to that which has been reported for rat lung, then naphthalene metabolism may potentially be influenced by progesterone receptor expression and it's effects on the enzymes involved either directly or through co-factor 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 difference stages of estrous with males found higher total P450 content, aniline hydroxylase (CYP2E), ethoxycoumarin O-deethylase (CYP2A) and aminopyrine N-demethylase activities in males than females at any stage (Watanabe et al. 1997). Total liver microsomal P450 content has also been found to be higher in male mice than female mice (Guo et al. 1993). Hepatic cytochrome c reductase is significantly lower at proestrous or diestrous compared to estrous in female rats (Watanabe et al. 1997). However, NADPH cytochrome c reductase activity in proestrus females was similar to males and was higher than that found in females in estrus or diestrus stages. Our study suggests possible hormonal regulation of the activity of enzymes involved in naphthalene bioactivation in the mouse lung. However, gene expression data suggests 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 different process regulate CYP2F2 gene expression in intra- vs extra- pulmonary airways. Further, female mice expressed significantly more CYP2F2 in their intrapulmonary airways than male mice correlating with increased cytoxicity 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. Further, the pattern of changes suggest 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 extent of toxicity or tumor formation, issues that may underlie the differential susceptibility of females to lung cancer.

ACKNOWLEDGEMENTS

The authors thank Dr. Suzette Smiley-Jewell for her comments on the manuscript, Collette Brown for technical assistance and Drs Charles Plopper and Alan Buckpitt for helpful advice.

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FOOTNOTES:

Financial support for this research: State of California Tobacco-Related Diseases Research Program Grant 11RT-0258, NIEHS grants ES04311, ES012720 and ES013066. UC Davis is an NIEHS Center for Environmental Health Sciences and we acknowledge Center support for core facilities used in this project (NIEHS ES05707).

Data was previously presented in abstract form at the 2004 Society of Toxicology Annual Meeting. LS Van Winkle, GL Baker, RL Stelck, CD Brown, CG Plopper and AR Buckpitt. Estrous Cycle alters Pulmonary Metabolism of Naphthalene (NA). *The Toxicologist.* Vol 78 (S-1) p 145 A#705.

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FIGURE LEGENDS:

Figure 1: Naphthalene is metabolized to isomeric epoxides the 1R, 2S and the 1S, 2R oxide. This is the obligate step in toxicity by naphthalene in the lung. The oxides can undergo conjugation with glutathione (catalyzed by glutathione transferases; GSH Tx) or be further metabolized via epoxide hydrolase to naphthalene dihydrodiol. 1-Napthol is generated spontaneously. The oxides or their downstream products (which may include further metabolism to several different quinones) are thought to mediate toxicity.

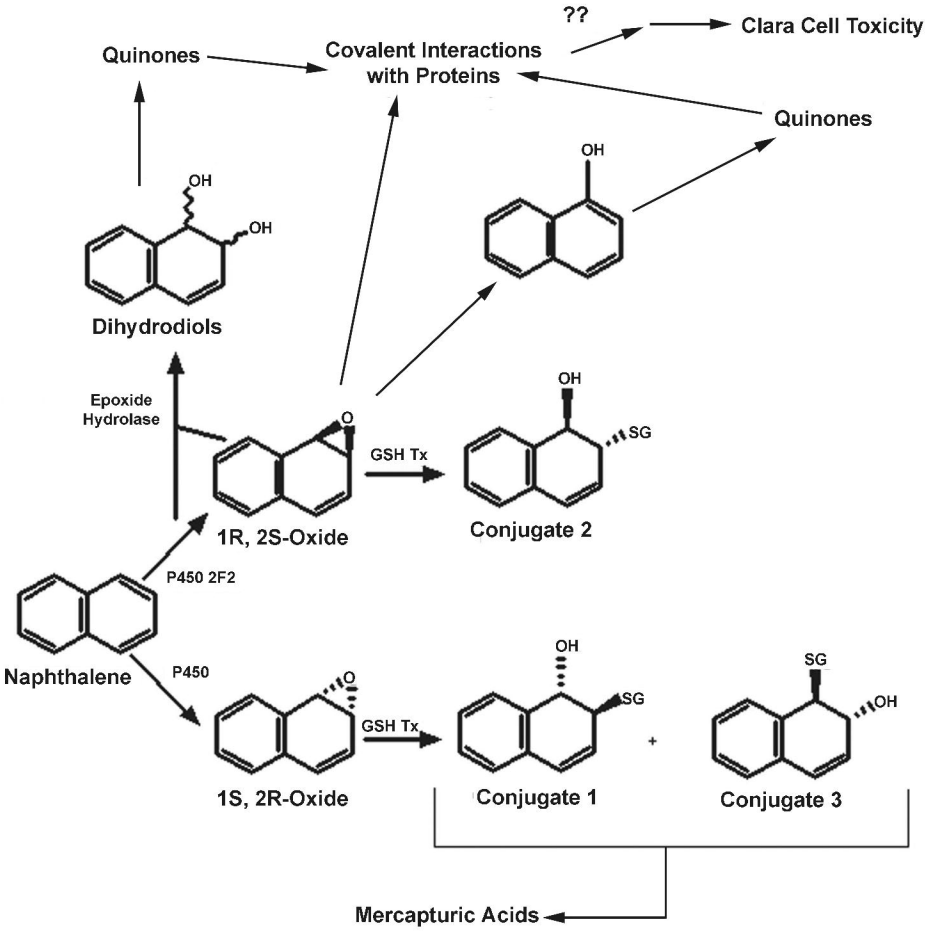
Figure 2- A. Examples of vaginal cytology found in the four estrous cycle stages. Mice in proestrus, estrus and noncycling (diestrus) were used for this study and were easily sorted into groups based on the relative abundance of cornified, basal, and inflammatory cells found in swabs. **B**. Serum levels of estradiol measured by ELISA in the serum of male and female mice. Estradiol levels differed significantly between proestrus and estrus cycle stages.

Figure 3- Comparison of the metabolism of naphthalene and percent of glutathione conjugate 2 and diol formed in dissected extrapulmonary airways (trachea and lobar bronchus) of female mice at various stages of estrous. **A**. Total metabolism of naphthalene, **B**. Glutathione conjugate 2 as a percent of total metabolism and **C**. naphthalene 1,2-dihydrodiol as a percent of total metabolism. * = significantly different from proestrus group; † = significantly different from estrus group: Significance accepted at $P < 0.05$.

Figure 4- Comparison of the metabolism of naphthalene and percent of glutathione conjugate 2 and diol formed in dissected intrapulmonary airways (bronchioles) of male mice with female mice at various stages of estrous. **A**. Total metabolism of naphthalene, **B**. Glutathione conjugate 2 as a percent of total metabolism and **C**. naphthalene 1,2-dihydrodiol as a percent of total metabolism. * = significantly different from proestrus group. § = significantly different from noncycling group. † = significantly different from estrus group. Significance accepted at $P < 0.05$.

Figure 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 noncycling group. † = significantly different from estrus group. Significance accepted at $P < 0.05$.

FIGURE 1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GSH Tx
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SG
1R, 2S-Oxide
Conjugate 2
P450 2F2
Naphthalene
P450
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1S, 2R-Oxide
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Mercapturic Acids

FIGURE 2

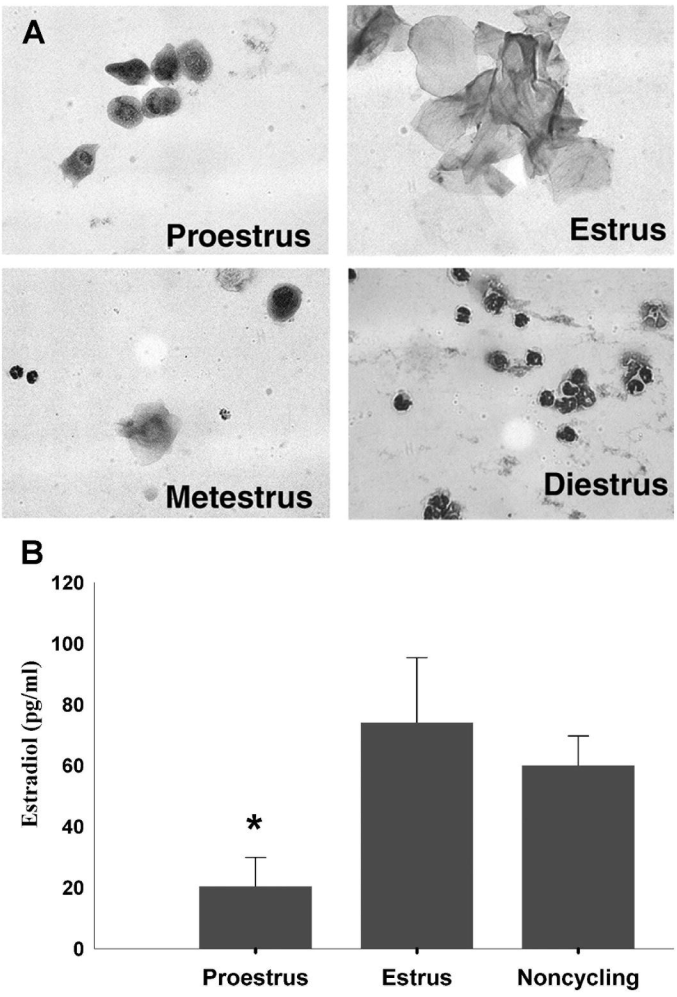

A
Proestrus
Estrus
Metestrus
Diestrus
B
Estradiol (pg/ml)
120
100
80
60
40
20
0
*
Proestrus
Estrus
Noncycling

FIGURE 3

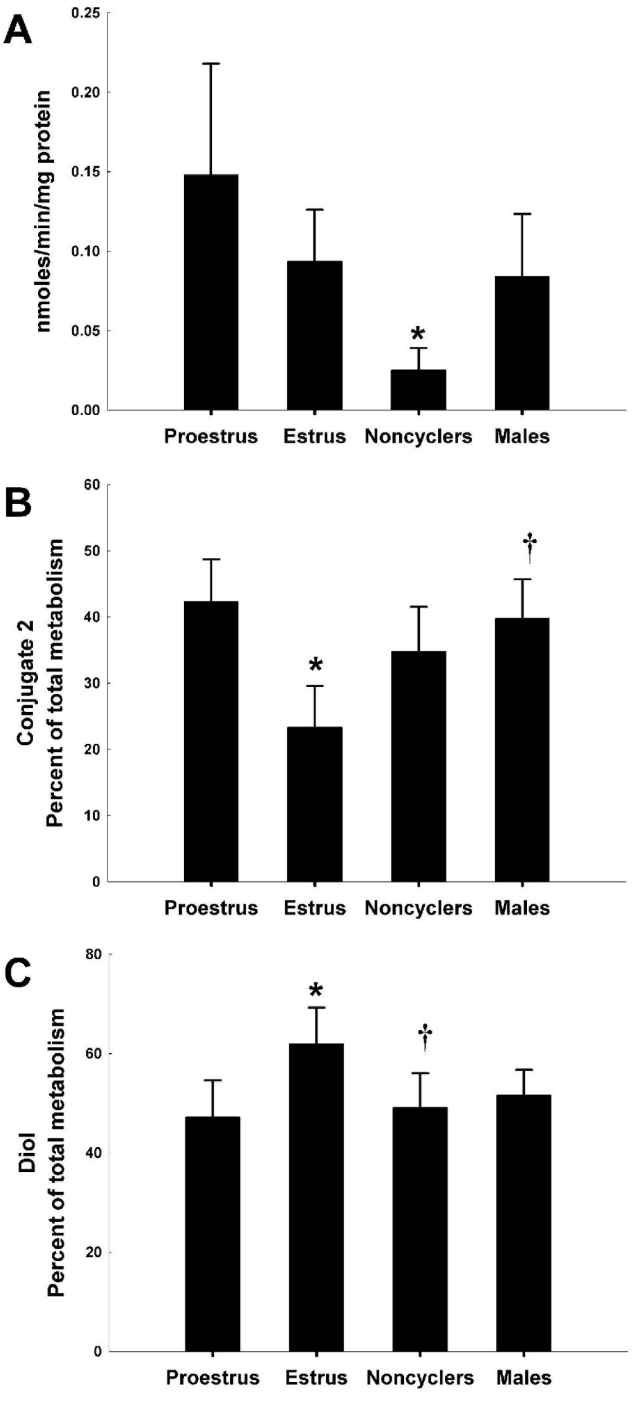

A
nmoles/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
*
Proestrus
Estrus
Noncyclers
Males
B
Conjugate 2
Percent of total metabolism
60
50
40
30
20
10
0
*
†
Proestrus
Estrus
Noncyclers
Males
C
Diol
Percent of total metabolism
80
60
40
20
0
*
†
Proestrus
Estrus
Noncyclers
Males

FIGURE 4

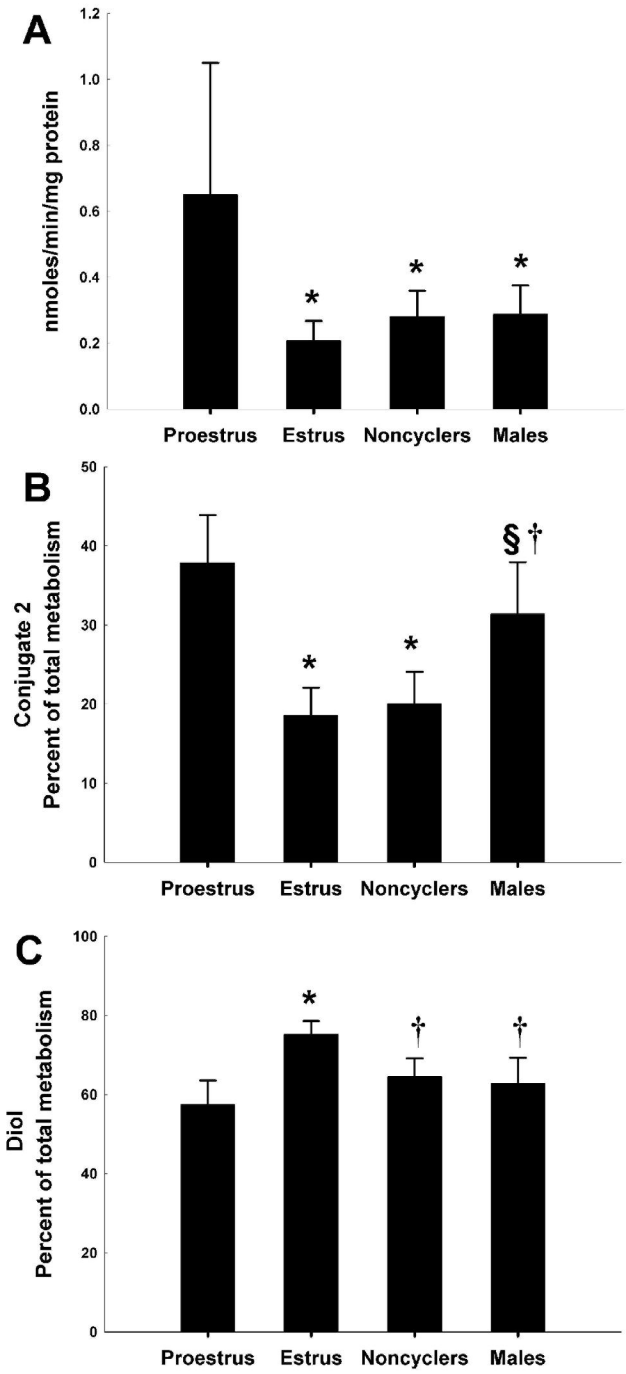
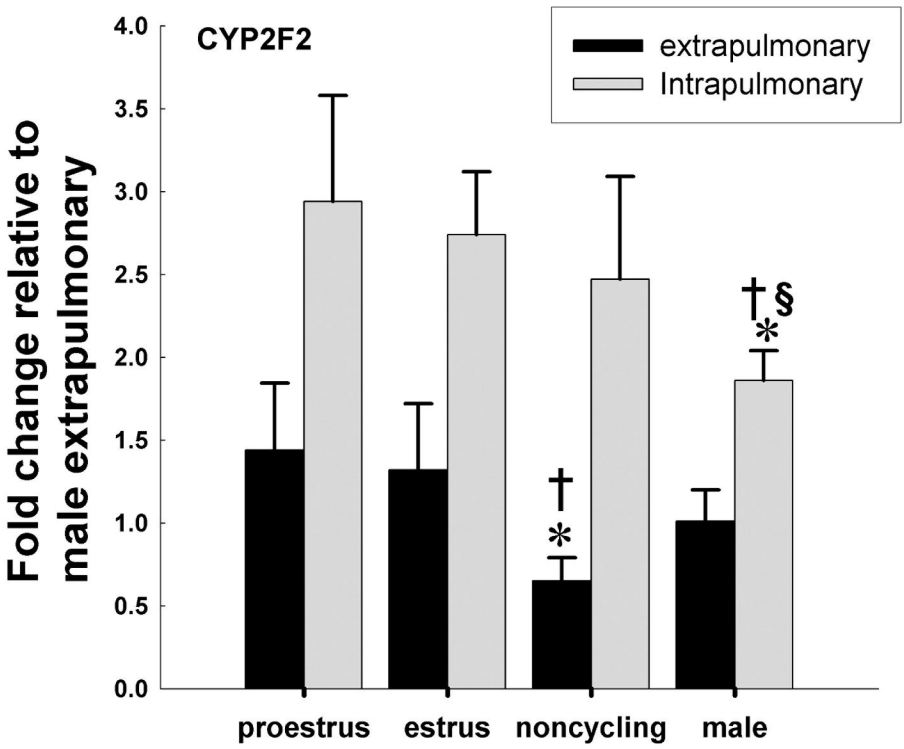

A
nmoles/min/mg protein
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Proestrus
Estrus
Noncyclers
Males
B
Conjugate 2
Percent of total metabolism
50
40
30
20
10
0
*
*
§ †
Proestrus
Estrus
Noncyclers
Males
C
Diol
Percent of total metabolism
100
80
60
40
20
0
*
†
†
Proestrus
Estrus
Noncyclers
Males

Figure 5


CYP2F2
extrapulmonary
Intrapulmonary
Fold change relative to male extrapulmonary
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
†
*
†§
*
proestrus
estrus
noncycling
male