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## Naphthalene cytotoxicity in microsomal epoxide hydrolase deficient mice

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### Abstract

Naphthalene (NA) is a ubiquitous pollutant to which humans are widely exposed. 1,2-Dihydro-1,2-dihydroxynaphthalene (NA-dihydrodiol) is a major metabolite of NA generated by microsomal epoxide hydrolase (mEH). To investigate the role of the NA-dihydrodiol and subsequent metabolites (ie 1,2-naphthoquinone) in cytotoxicity, we exposed both male and female wild type (WT) and mEH null mice (KO) to NA by inhalation (5, 10, 20 ppm for 4 hours). NA-dihydrodiol was ablated in the KO mice. High-resolution histopathology was used to study site-specific cytotoxicity, and formation of naphthalene metabolites was measured by HPLC in microdissected airways. Swollen and vacuolated airway epithelial cells were observed in the intra- and extrapulmonary airways of all mice at and below the current OSHA standard (10 ppm). Female mice may be more susceptible to this acute cytotoxicity. In the extrapulmonary airways, WT mice were more susceptible to damage than KO mice, indicating that the metabolites associated with mEH-mediated metabolism could be partially responsible for cytotoxicity at this site. The level of cytotoxicity in the mEH KO mice at all airway levels suggests that non-mEH metabolites are contributing to NA cellular damage in the lung. Our results indicate that the apparent contribution of mEH-dependent metabolites to toxicity differs by location in the lung. These studies suggest that metabolites generated through the mEH pathway may be of minor importance in distal airway toxicity and subsequent carcinogenesis from NA exposure.

### Keywords

cytochrome P450; cell injury/cell death; carcinogen metabolism; microsomal epoxide hydrolase; HPLC; lung/pulmonary/olfactory; toxicology

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### AUTHORSHIP CONTRIBUTIONS

*Participated in research design:* Morin, Buckpitt, and Van Winkle

*Conducted experiments:* Carratt, Morin, Buckpitt, Edwards, Van Winkle

*Performed data analysis:* Carratt and Morin

*Wrote or contributed to the writing of the manuscript:* Carratt, Buckpitt, and Van Winkle

## INTRODUCTION

Naphthalene (NA) is a widespread pollutant that is currently classified as a possible human carcinogen, though this classification is under review (IARC, 2002; USEPA, 1998). The results of the NTP (National Toxicology Program) chronic rodent bioassays for NA found significant, dose-dependent increases in alveolar adenomas in the lungs of female mice, and in adenomas and neuroblastomas of the nasal epithelium in rats (Abdo et al. 1992; 2001). Primary sources of NA include biomass burning (Kakareka and Kukharchyk, 2003), automobile emissions (USEPA, 1986) and tobacco smoke (Schmeltz et al., 1976; Witschi et al., 1997); however, NA has also been found in food (Kobayashi et al., 2008) and ground water. In the National Human Adipose Tissue Survey, 40% of the study population had detectable levels of NA in adipose tissue (Stanley, 1986). Another study found that 75% of a lactating female population had measurable levels of NA in milk (Pellizzari et al., 1982).

The toxicity of NA is cytochrome P450 dependent, with reactive metabolites produced by enzymes in both the lung and the liver (Buckpitt and Warren, 1983). In the lung, nonciliated bronchiolar Club (formerly known as Clara) cells express the highest amounts of P450 (Plopper et al., 1987) and are severely affected by NA toxicity (Buckpitt et al., 1995). Recurrent cycles of cytotoxicity and proliferation are thought to be the driving force behind formation of mouse lung tumors (National Toxicology Program, 1992; West et al., 2001) and rat nasal tumors (Long et al., 2003; 2000) following chronic exposure to NA because tumors form in respiratory tissues that exhibit high acute toxicity. However, it is unknown which metabolite(s) drive(s) this toxicity. A summary of NA metabolism to potentially toxic metabolites is diagrammed in Fig. 1.

In mice, the bioactivation of NA by P450 enzymes (CYP2A5 and CYP2F2) generates a 1,2-epoxide (Li et al., 2011). Glutathione (GSH) conjugates can form from the epoxide, and are eliminated in urine as mercapturic acids (Pakenham et al., 2002). NA 1,2-epoxide can also be metabolized by microsomal epoxide hydrolase (mEH) to NA-dihydrodiol (Kitteringham et al., 1996). When GSH is depleted, it is possible that the mEH pathway may be favored. Previous studies demonstrated that severe GSH depletion increases NA toxicity in the respiratory tract (Phimister et al., 2004; Plopper et al., 2001). It has been speculated that this increased toxicity and the resulting tumorigenesis of NA is mediated by the formation of a 1,2-naphthoquinone (NQ) through the mEH pathway. In fact, Bogen et al. suggests that the most recent EPA evaluation of NA hinged on the interpretation of bioassays that suggested that 1,2-NQ is genotoxic (Bogen et al., 2008). However, the toxicity could also result from increased levels of NA epoxide. 1,2-NA epoxide can form adducts with protein (Waidyanatha et al., 2002). In animal models, the extent to which specific metabolic pathways (to epoxides, quinones, and diols) contribute to cytotoxicity is unknown. Studies indicating the potential genotoxicity of NQs are either in vitro (Flowers-Geary et al., 1996; National Toxicology Program, 1992) or have not evaluated critical tumor sites, such as the lung. Recent studies (Saeed et al. 2007; Saeed et al. 2009) demonstrated the formation of both depurinated and stable NA DNA adducts in vitro and in skin painting studies but the relevance of the painting studies is uncertain because skin is not a known target tissue for NA cytotoxicity. NA metabolite-derived DNA adducts have not been demonstrated in the respiratory system of any species yet. There is a current need for information relating

specific *in vivo* NA metabolism pathways to NA toxicity so that risk from exposure can be understood.

In the current study, we investigated the role of mEH-mediated metabolites (ie 1,2-NQ) in cytotoxicity. To do this, we used mEH knockout (KO) mice (Miyata et al., 1999). These mice have been used previously to investigate the role of mEH in benzene-induced toxicity (Bauer et al., 2003) and 7, 12-dimethylbenz[a]anthracene-induced tumorigenicity (Miyata et al., 1999). The aims of this study were to determine (1) if mEH metabolites are of importance in the overall toxicity of NA at known tumor sites, (2) if cytotoxicity of NA is dose-dependent in mEH KO mice, and (3) if NA toxicity differs between intra- and extrapulmonary airways in mEH KO mice. To explore these questions, we exposed both male and female wild type (WT) and mEH null mice (KO) to NA by inhalation (5, 10, 20 ppm; 4 hrs). These data will inform future studies of NA risk assessment.

## METHODS

### Animals

For these experiments, adult (8-10 week old) male and female B6:129 wild type (WT) and mEH1 null mice (KO) were obtained from Jackson labs and re-derived and maintained by the mouse biology program at UC Davis from a breeding stock initially created by Frank J. Gonzales at the National Institute of Health (Miyata et al., 1999). All mice were maintained in a barrier facility, housed in a high efficiency particle air (HEPA)-filtered cage rack in AAALAC approved facilities on a 12-hour light/dark cycle with food and water *ad libitum*. All animal experiments were performed under protocols approved by the University of California Davis IACUC in accordance with National Institutes of Health guidelines.

### *Ex vivo* metabolite formation in airways

Lungs from WT and KO naïve male ( $n_{WT}=5$ ,  $n_{KO}=6$ ) and female ( $n_{WT}=5$ ,  $n_{KO}=5$ ) mice were cannulated, removed *en bloc*, inflated with agarose and microdissected (Plopper et al., 1991). Trachea and lobar bronchi were separated and defined as extrapulmonary airways. Airways microdissected from the lobes of the lung were defined as intrapulmonary airways. Extra- and intrapulmonary airways were then placed in 1 ml Waymouth's medium on ice with 5-10 CDNB units glutathione transferase (GST), 5 mM GSH, 250  $\mu$ M NA dissolved in 5  $\mu$ l HPLC grade methanol. Reaction vessels were capped tightly and transferred to a shaking water bath at 37° C for 120 min. Following the incubation, samples were transferred to an ice bath and 1 ml ice-cold HPLC grade methanol was added to quench the reaction. Samples were homogenized and then centrifuged at 16000  $\times$  G for 10 min to remove precipitated proteins. The supernatant was transferred to a clean tube and evaporated under vacuum to complete dryness. Dried samples were stored at -80°C until HPLC analysis. The protein pellet was dissolved in 1 N NaOH and an aliquot was removed for determination of protein, using a bovine serum albumin standard for protein determinations (Lowry et al., 1951).

### HPLC separation of NA metabolites

Samples were reconstituted in 100 µl water and centrifuged to remove particulates. NA-dihydrodiol and glutathione conjugates of 1,2-epoxide were separated on a Phase Sep C<sub>18</sub> ODS2 column (250 × 4.6 mm i.d.; 5-µm particle) (Waters, Milford, MA). The eluates were monitored by UV absorbance at 260 nm as described previously (Buckpitt et al., 1987). Metabolites, prepared synthetically, were used to generate standard curves.

### High-resolution light microscopy

Male and female mice (n=3) were exposed to filtered air +/- NA vapor at 0, 5, 10 or 20 ppm for 4 hours as described previously (West et al., 2001). All animals were necropsied 24 hrs after the whole body exposure. At necropsy, animals were euthanized with an overdose of pentobarbital and the trachea was cannulated. The lungs were removed *en bloc* and fixed via tracheal cannula at 30 cm of pressure with Karnovsky's fixative (0.9% glutaraldehyde/0.7% paraformaldehyde in cacodylate buffer, adjusted to pH 7.4, 330 mOsmol/kgH<sub>2</sub>O) for 1 hr. Lungs were stored in fixative until use. Karnovsky's fixed tissue samples were embedded separately in Araldite 502 epoxy resin. Specimens were sectioned at 1 µm (Leica Ultracut UCT ultramicrotome with glass knives) and stained with Methylene Blue/Azure II. High magnification images were captured using a 20× objective lens using an Olympus BH-2 microscope in bright field mode.

Tissues were evaluated using a damage score matrix without knowledge of exposure groups. Terminal bronchioles were scored for intrapulmonary toxicity, and trachea was scored for extrapulmonary toxicity. Extrapulmonary airway damage scores were as follows: 0 – no damage, 1 – few swollen/vacuolated cells, 2 – swollen cells and cells with vacuoles throughout cytoplasm, 3 – all cells have vacuoles throughout cytoplasm. Intrapulmonary scores were: 0 – no damage, 1 – damage only in the proximal airway, 2 - damage in proximal airway and a few swollen cells in terminal bronchioles, 3 – damage in proximal airway and many swollen/vacuolated cells in terminal bronchioles.

### Gene Expression

The trachea was cannulated and the lungs were removed and inflated to capacity with RNAlater (Ambion/Applied Biosystems; Foster City, CA). The lungs (n =5) were stored at 4°C for 24 hr and then moved to 22°C until microdissection and RNA isolation could be performed. Intrapulmonary airways were microdissected from the surrounding parenchyma. RNA was reverse transcribed into single-stranded cDNA using the High-Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's suggested protocol. qRT-PCR was carried out in the StepOnePlus Real-Time PCR System.

### Statistics

Data are reported as mean ± standard deviation unless otherwise stated. Parametric data analysis was performed using Student's unpaired t-test. Values of p < 0.05 were considered statistically significant. Statistical analysis was performed using StatView 5.0.1 (SAS Institute Inc., 1999).

## RESULTS AND DISCUSSION

### Naphthalene metabolism

The first step of NA metabolism is the conversion to 1,2-epoxide by cytochrome P450 monooxygenase (Fig. 1). The majority of 1,2-epoxide is detoxified by conjugation to GSH and eliminated as mercapturic acids in urine (Habig et al., 1974), converted to 1,2-dihydrodiol by mEH, or rearranges to 1-naphthol which is eliminated as glucuronide or sulfate conjugates in the urine. NA-GSH conjugates can be measured by HPLC, but 1,2-NQ and 1,2-epoxide are unstable and difficult to measure directly. Therefore, we assessed the P450 mediated metabolism of NA to the 1,2-epoxide by measuring the four glutathione conjugates and the 1,2-dihydrodiol, the measurable precursor to 1,2-NQ, as in previous studies of NA metabolism (Buckpitt et al., 1995). We confirmed that NA-dihydrodiol was ablated in the KO mice for both intrapulmonary and extrapulmonary airways, for both sexes (Fig. 2). Total metabolism is defined in Fig. 2 as the sum of the five measured metabolites. The significant decrease in dihydrodiol resulted in a significant decrease in total metabolism for both airways and sexes in the KO mice.

As seen in these figures, GSH conjugates are not formed in equal amounts. In WT and KO mice, conjugate 2 is the primary NA metabolite, followed in abundance by the dihydrodiol. In KO mice, GSH conjugate 2 was significantly decreased for male mice ( $p = 0.015$ ) in extrapulmonary airways but not in intrapulmonary airways, or for female mice. Besides the clear differences in dihydrodiol generation, differences in the rates of GSH conjugate 2 formation was the only difference detected between the WT and KO mice, and this was only noted in male mice. Reasons for this decrease are unclear, but this decrease in the detoxifying GSH conjugate does not appear to correlate with increased susceptibility to cytotoxicity in males.

In the extrapulmonary airways, WT mice had significantly total higher metabolism than KO mice for both males ( $p = 0.016$ ) and females ( $p = 0.017$ ). The only observed sex difference was with conjugate 3, which was higher in male KO mice than in female KO mice ( $p = 0.034$ ). In the intrapulmonary airways, the males had higher metabolism for many of the measured metabolites (Table 1). These sex differences in intrapulmonary metabolism are likely not a result of our KO model, as the majority of statistically significant sex differences also exist in the WT mice. We measured statistically higher total metabolism for males than females in intrapulmonary airways, for both KO ( $p < 0.001$ ) and WT ( $p = 0.023$ ).

In the lung, NA activation to a 1,2-epoxide is driven by the high levels of *Cyp2f2* in nonciliated bronchiolar Club cells (Plopper et al., 1987). To confirm that the differences in intrapulmonary metabolism were not a result of altered *Cyp2f2* expression, we measured gene expression in microdissected airways. *Cyp2f2* expression was not altered in the KO mouse, however the expression of *Cyp2f2* is higher in female mice relative to male mice (Fig. 3B). CC10 (club cell 10-kDa protein) expression was increased in the male KO mouse, indicating male KO mice might have more club cells than WT mice ( $p = 0.036$ , Fig. 3A).

## Cytotoxicity

The dose and route of exposure are important in predicting NA toxicity. Previous studies have found that inhalation of NA vapor below the current OSHA standard of 10 ppm causes proximal airway Club cell injury in mice (West et al., 2001). However, when a similar dose is administered intraperitoneally, injury is primarily observed in the distal airways, including terminal bronchioles (West et al., 2001). At intraperitoneal doses up to 200 mg/kg, the bronchi and trachea can contain affected cells, although the extent of injury varies by mouse strain (Lawson et al., 2002) and sex (Van Winkle et al., 2002).

All mice in this experiment were sensitive to damage from NA exposure; however, the extent of cytotoxicity varied by airway generation and exposure concentration (Fig. 4). Smaller intrapulmonary airways experienced slight cytotoxicity at and below the OSHA standard of 10 ppm. Our results indicate that damage to the large extrapulmonary airways can occur at exposure concentrations as low as 5 ppm when exposed acutely to NA. For the extrapulmonary airways, WT cells appear to be more susceptible than KO at 5 and 10 ppm. We observed severe WT tissue damage at 10 ppm, with a greater degree of vacuolization and swelling in females. This same pattern exists for the KO mice at 10 ppm, however the overall damage is less severe than in WT mice. At 20 ppm, swollen/vacuolated airway epithelial cells were observed in the extra- and intrapulmonary airways of all mice at 20 ppm, though this effect was minimal in WT male extrapulmonary airways.

In the extrapulmonary airways, cytotoxicity may be partially a result of mEH downstream metabolites (Fig. 4AB). However, these results do indicate that non-mEH metabolites are contributing to NA cellular damage in the lung. There appears to be a dose-dependent response to NA exposure in KO mice, while WT mice are very sensitive to damage even at 5 ppm. In the critical tumor site, intrapulmonary airways were more severely damaged in KO than WT for 20 ppm (Fig. 4CD). Toxicity cannot be from the 1,2-NQ in the intrapulmonary airways, as that pathway has been ablated in the KO animals.

We observed a possible increase in intrapulmonary cytotoxicity at 20 ppm in WT female relative to WT males. In the extrapulmonary airways, we observed a possible increase in cytotoxicity to KO females relative to KO males at 10 ppm. Representative images from female and male extrapulmonary airways after acute 10 ppm exposure are shown in Fig. 5. In both extrapulmonary and intrapulmonary airways, the pattern of cytotoxicity suggests increased susceptibility to acute NA exposure in females. This is consistent with previous studies. The NTP chronic bioassay showed that female mice—but not male mice—will develop lung tumors following chronic NA exposure (National Toxicology Program, 1992).

Our results indicate that the apparent contribution of mEH-dependent metabolites to toxicity differs by location in the lung. For the extrapulmonary airways, WT cells are more susceptible than KO at 5 and 10 ppm. This indicates that cytotoxicity in this region may be partially a result of mEH downstream metabolites, including the 1,2-NQ. However, because the KO animals are still susceptible to extrapulmonary damage from NA exposure, mEH is not the only enzyme driving this toxicity. In the intrapulmonary airways, where mouse lung tumors are known to form following inhalation of NA vapor (Abdo et al., 2001), cells from KO mice are more susceptible to NA injury than WT at 20 ppm. This observed toxicity

cannot be from the 1,2-NQ, as that pathway has been ablated in the KO animals. Further investigation is needed to explore the *in vivo* toxicity of the mEH pathway, to determine if either (1) metabolites generated through the mEH pathway are of minor importance in the overall toxicity and subsequent distal airway carcinogenesis from NA exposure or that (2) compensatory alterations in gene expression associated with mEH KO may alter other pathways in the activation and detoxification of NA.

## Conclusions

Sex, airway generation and the mEH pathway affect susceptibility to cytotoxicity in mice, following acute exposure to NA. Females may be more susceptible to NA toxicity than males, and in the intrapulmonary airways, this may be due in part to sex differences in GSH conjugate formation. In the extrapulmonary airways, WT mice were more susceptible to damage than KO mice, indicating mEH could be partially responsible for cytotoxicity at this site. In the intrapulmonary airways, metabolites generated through the mEH pathway may be of minor importance in distal airway toxicity and subsequent carcinogenesis from NA exposure. The pattern of toxicity observed in the KO mice suggests that mEH metabolites may not be driving the overall toxicity of NA for known tumor sites. The 1,2-NQ is often suspected of being the toxic metabolite of NA; however, our results suggest that other metabolites, perhaps a NA-epoxide or the 1,4-NQ, or a pathway that is currently unknown, may be contribute to intrapulmonary toxicity and subsequent carcinogenesis.

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## Abbreviations used

<b>P450</b>	cytochrome P450
<b>NA-dihydrodiol</b>	1,2-dihydro-1,2 dihydroxynaphthalene
<b>GSH</b>	glutathione
<b>GST</b>	glutathione transferase
<b>KO</b>	knockout
<b>mEH</b>	microsomal epoxide hydrolase
<b>NA</b>	naphthalene
<b>NQ</b>	naphthoquinone
<b>OSHA</b>	Occupational Safety and Health Administration
<b>WT</b>	wild type



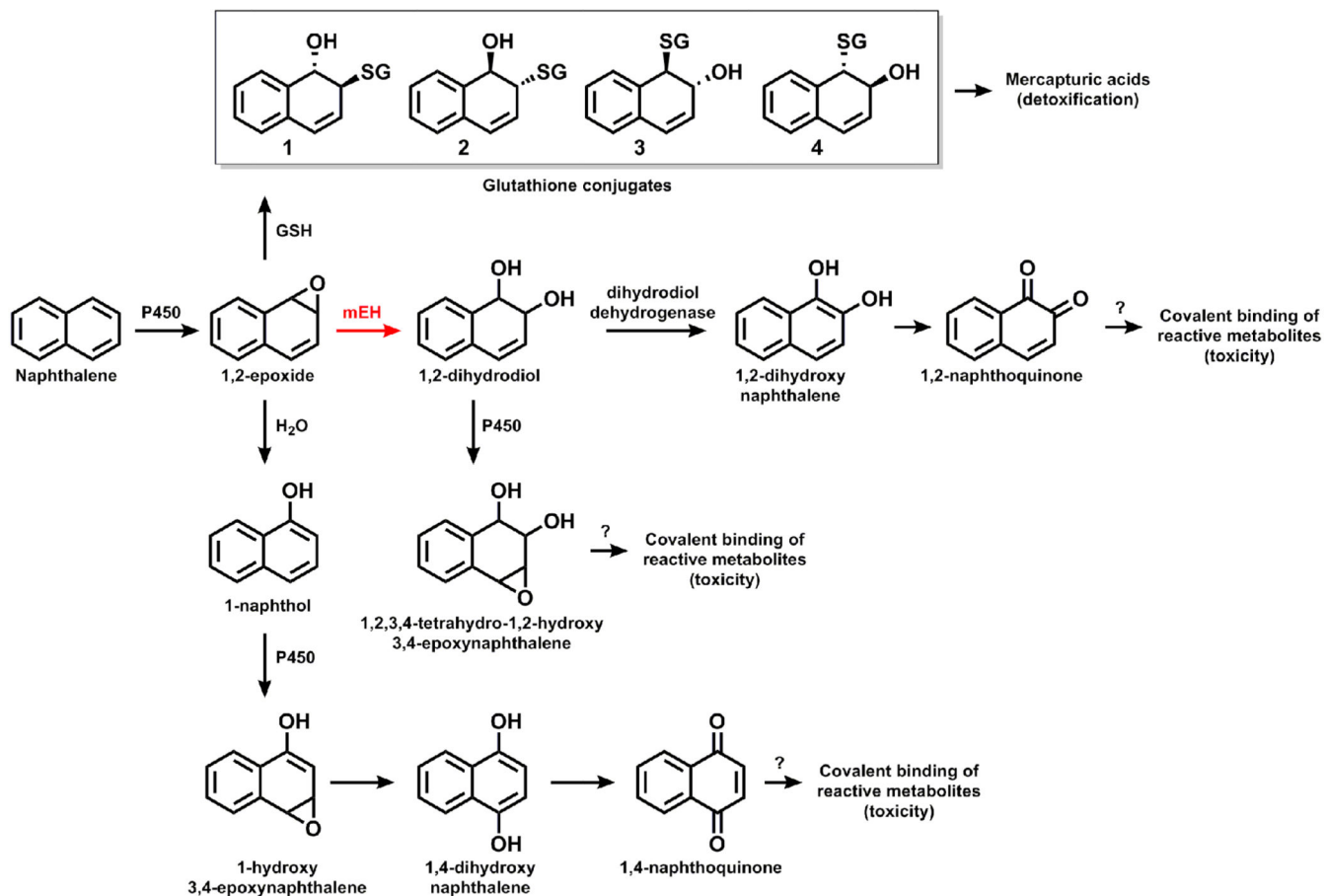
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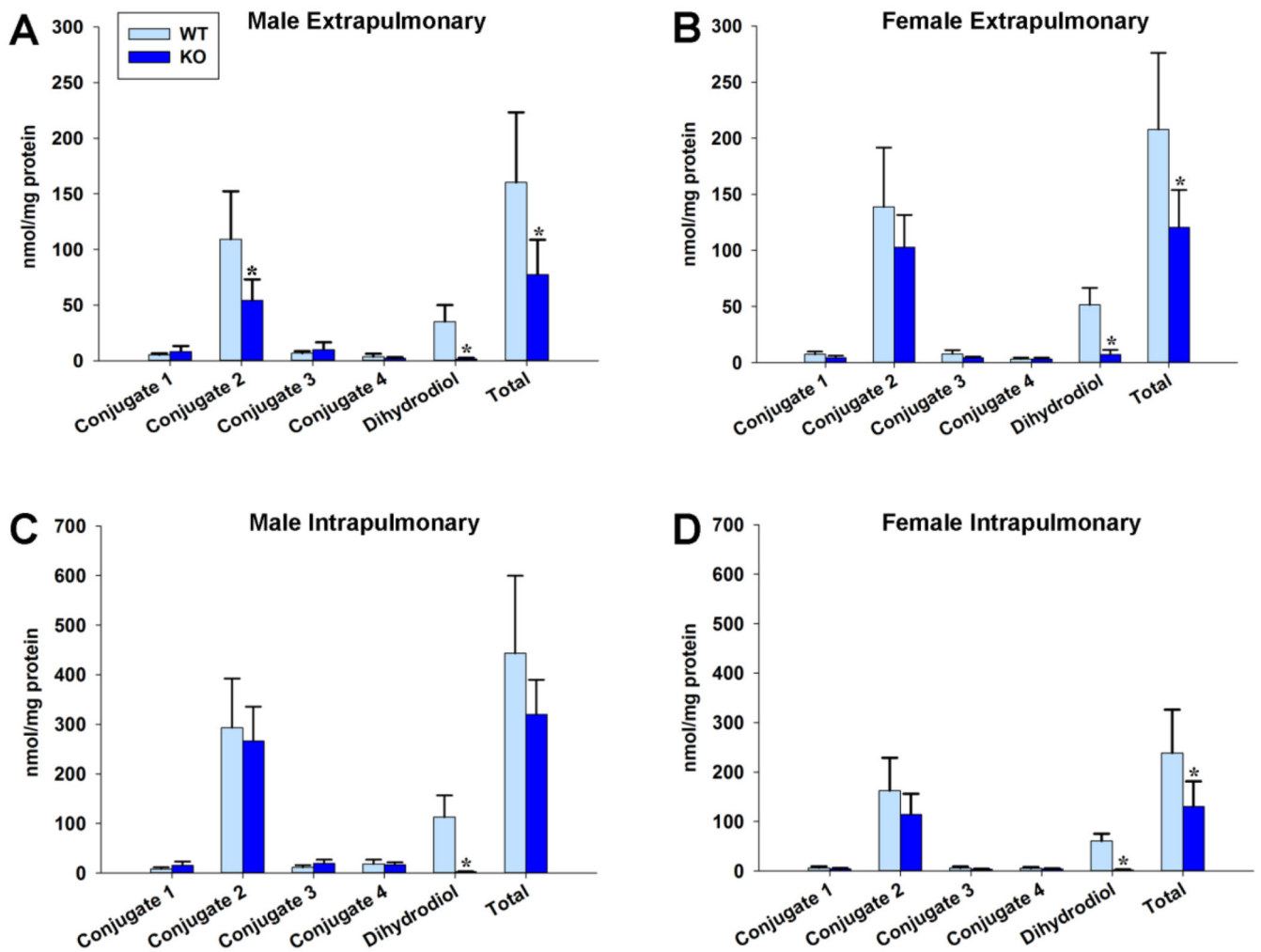
### Highlights

- mEH metabolites may not be driving the overall toxicity of NA for known tumor sites
- The mEH pathway may be partially responsible for cytotoxicity in extrapulmonary airways
- Acute exposure to naphthalene causes cytotoxicity in all airway generations at 5 ppm
- Female mice may be more susceptible to naphthalene acute toxicity than males



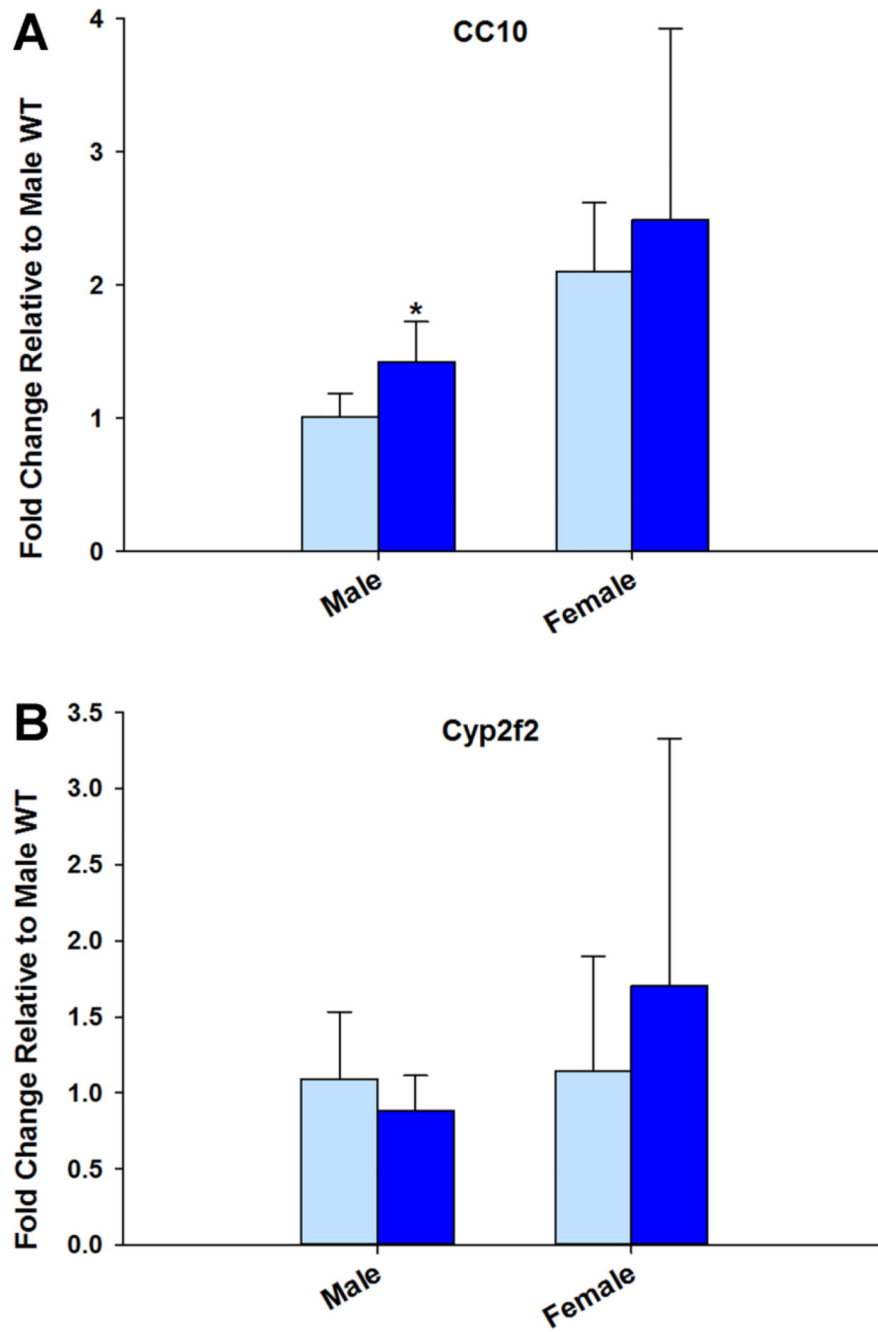
**Fig. 1. Naphthalene metabolism to plausible toxic metabolites**

Quantifiable metabolites are the GSH conjugates, 1,2 NA-dihydrodiol, and 1-naphthol. GSH conjugates are usually associated with detoxification while the mEH pathway is associated with toxicity.

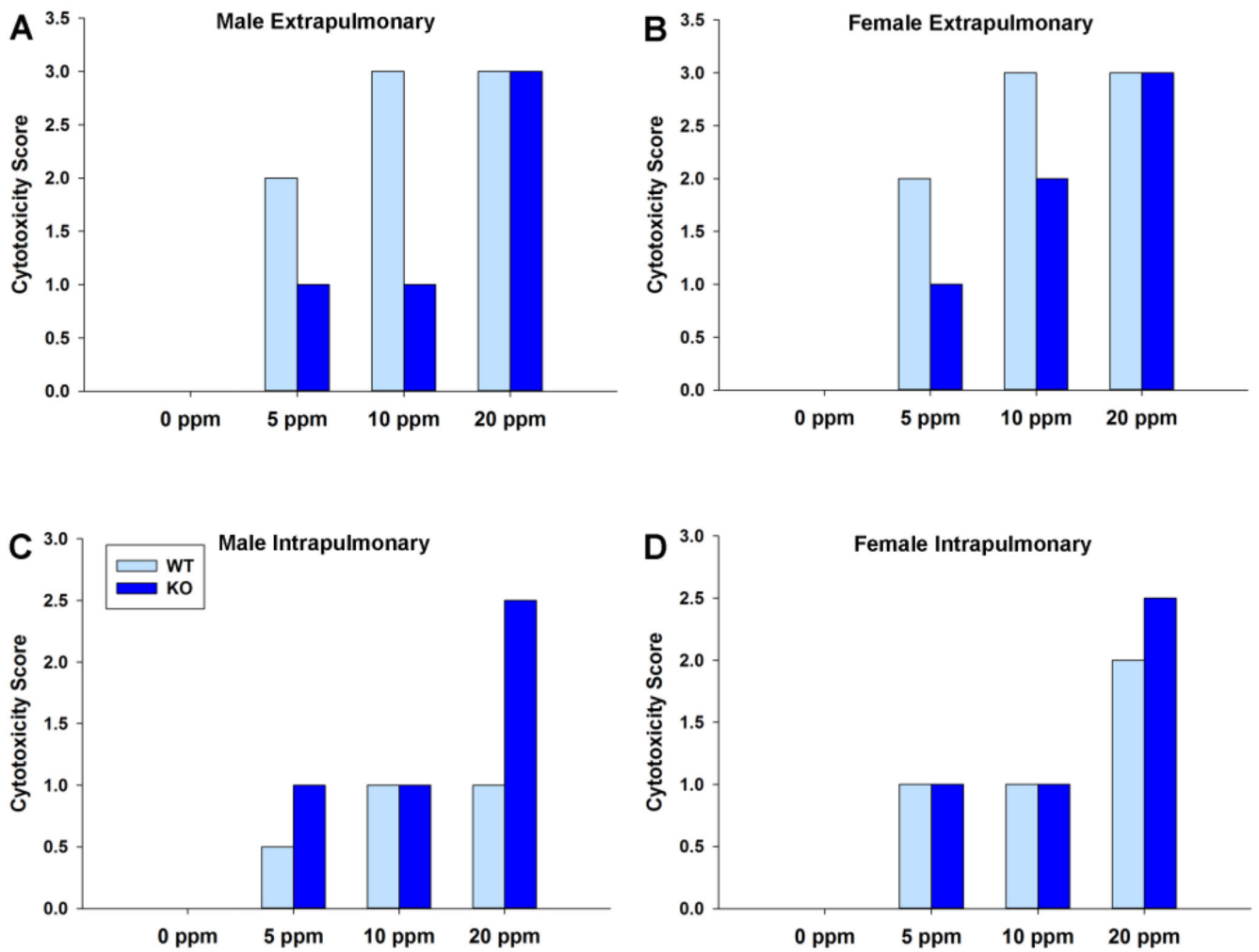


**Fig. 2. Metabolism to the 1,2-dihydrodiol is ablated in mEH KO mice**

This verifies the KO mouse is not metabolizing significant epoxide through the mEH pathway in these airways: (A) male extrapulmonary, (B) female extrapulmonary, (C) male intrapulmonary, (D) female intrapulmonary. \* denotes significant difference ( $p < 0.05$ ) from WT

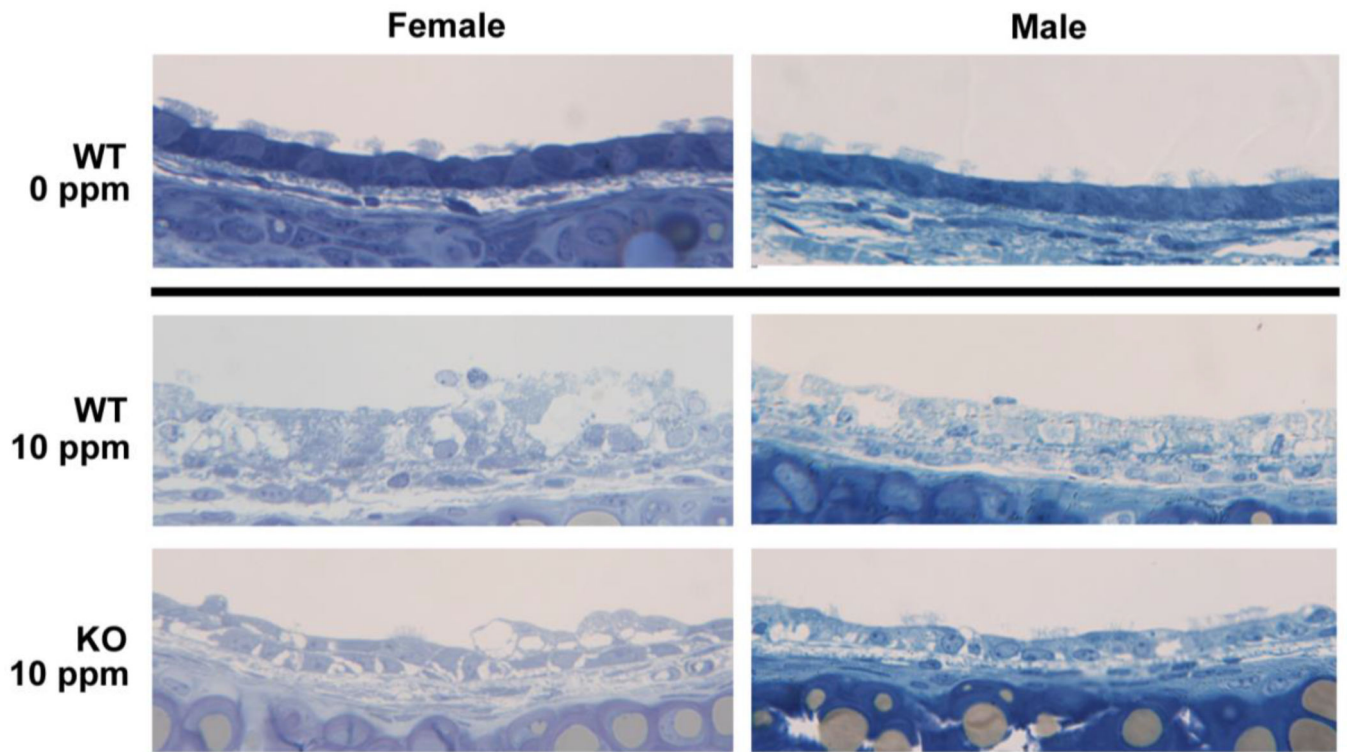


**Fig. 3. Differences in airway metabolism were not a result of decreased *Cyp2f2* or CC10 expression**  
Male CC10 fold change is greater for KO than WT. Fold change expressed relative to WT. \* denotes significant difference ( $p < 0.05$ ) from WT



**Fig. 4. KO mice had more severely damaged airways at sites of known NA-driven tumor formation**

Male and female differences appear to be present in KO extrapulmonary airways at 10 ppm and in WT intrapulmonary airways at 20 ppm.



**Fig. 5. Extrapulmonary toxicity may be more severe for females than males at 10 ppm**  
Representative trachea sections illustrating severe WT tissue damage at 10 ppm, with a greater degree of vacuolization and swelling in the female section. There is no damage at 0 ppm for WT or KO mice (only WT shown).



**Table 1**  
**Sex differences in intrapulmonary NA metabolism are not caused by mEH KO**

We measured statistically higher metabolism in control male intrapulmonary airways for most endpoints ( $p < 0.05$ ). Data are expressed as the mean  $\pm$  SD for  $n = 5-6$  as nmoles metabolite/mg protein/120 minutes.

	Wildtype			Knockout		
	Male	Female	P-value	Male	Female	P-value
Conjugate 1	8.10 $\pm$ 3.38	6.30 $\pm$ 3.02	NS	15.2 $\pm$ 7.91	3.70 $\pm$ 1.83	0.006
Conjugate 2	293 $\pm$ 99.2	162 $\pm$ 66.3	0.022	267 $\pm$ 69.2	114 $\pm$ 41.7	0.001
Conjugate 3	11.3 $\pm$ 4.50	5.69 $\pm$ 3.04	0.028	19.1 $\pm$ 7.72	3.49 $\pm$ 0.690	0.002
Conjugate 4	18.2 $\pm$ 8.43	5.47 $\pm$ 2.33	0.019	16.7 $\pm$ 4.79	4.20 $\pm$ 0.830	< 0.001
Dihydrodiol	113 $\pm$ 43.5	60.4 $\pm$ 14.9	0.025	2.43 $\pm$ 0.890	2.04 $\pm$ 0.580	NS
Total	443 $\pm$ 156	239 $\pm$ 87.5	0.023	320 $\pm$ 70.0	130 $\pm$ 51.2	< 0.001

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