Essential role of the cytochrome P450 enzyme CYP2A5 in olfactory mucosal toxicity of naphthalene

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ABBREVIATIONS: P450, cytochrome P450; NA, naphthalene; GSH, reduced glutathione; NA-GSH, naphthalene-glutathione; AP-GSH, acetaminophen-glutathione; B6, C57BL/6; WT, wild type; NPSH, nonprotein sulfhydryl; OM, nasal olfactory mucosa; LC-MS, liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; AUC, area under the concentration-time curve; Cmax, maximal plasma concentration; Tmax, time when Cmax is reached; t1/2, elimination half-time.
Abstract

Naphthalene (NA), a ubiquitous environmental pollutant that can cause pulmonary and nasal toxicity in laboratory animals, requires cytochrome P450 (P450)-mediated metabolic activation in order to cause toxicity. Our recent study using a Cyp2f2-null mouse showed that CYP2F2 plays an essential role in NA-induced lung toxicity, but not in NA-induced nasal toxicity. The aim of this study was to determine whether mouse CYP2A5, abundantly expressed in nasal olfactory mucosa (OM) and liver, but less in lung, plays the major role in the bioactivation and toxicity of NA in the OM. We found, by comparing Cyp2a5-null and wild-type (WT) mice, that the loss of CYP2A5 expression led to substantial decreases in rates of NA metabolic activation by OM microsomes. The loss of CYP2A5 did not cause changes in systemic clearance of NA (at 200 mg/kg, i.p.). However, the Cyp2a5-null mice were much more resistant than were WT mice to NA-induced nasal toxicity (though not lung toxicity), when examined at 24 h after NA dosing (at 200 mg/kg i.p.), or to NA-induced depletion of total nonprotein sulfhydryl in the OM (though not in the lung), examined at 2 h after dosing. Thus, mouse CYP2A5 plays an essential role in the bioactivation and toxicity of NA in the OM, but not in the lung. Our findings further illustrate the tissue-specific nature of the role of individual P450 enzymes in xenobiotic toxicity, and provide the basis for a more reliable assessment of the potential risks of NA nasal toxicity in humans.
Introduction

Naphthalene (NA), a component of coal tar, crude oil and cigarette smoke and a chemical intermediate for the manufacturing of numerous commercial products, is a ubiquitous environmental pollutant (Preuss et al., 2003; Riviere et al., 1999; McDougal et al., 2000). NA exposure can cause tissue-specific toxicity to Clara cells in mouse lung and to epithelial cells in the nasal olfactory mucosa (OM) of rats and mice (Plopper et al., 1992). NA is carcinogenic in both rat and mouse (Abdo et al., 1992, 2001), and it has been classified as a potential human carcinogen by International Agency for Research on Cancer (IARC, 2002). The mechanism of NA’s carcinogenicity is believed to involve repeated cycles of epithelial injury and repair, in connection with NA bioactivation and the resulting acute cytotoxicity (Brusick, 2008).

The bioactivation of NA is mainly catalyzed by cytochrome P450 (P450) enzymes, which produce reactive NA-1,2-epoxide. The epoxide can conjugate with reduced glutathione (GSH), form adducts with sulfhydryl groups in cellular proteins, or be further converted to reactive quinones, diepoxides and diol epoxides (Buckpitt et al., 2002). Rapid formation of NA oxide and quinones can lead to GSH depletion, increased cellular oxidative stress, and eventually cell death. The high abundance of CYP2F2, a highly active and efficient NA oxidase, in the Clara cells of mouse lung has been proposed to explain the high sensitivity of these cells to NA’s cytotoxicity (Buckpitt et al., 1995). The latter hypothesis was confirmed recently by the finding that Cyp2f2-null mice are resistant to NA’s lung toxicity (Li et al., 2011). However, although CYP2F2 is also expressed in the OM, Cyp2f2-null mice were not protected against NA’s toxicity in the OM, a result suggesting a more prominent role by other P450 enzymes expressed in the OM (Li et al., 2011).

The aim of this study was to test the hypothesis that CYP2A5, an abundant P450 enzyme expressed in mouse OM (Gu et al., 1998), plays an important role in NA toxicity in the OM. CYP2A5 is active toward numerous drugs and other xenobiotic compounds (Su and Ding, 2004; Wong et al., 2005; Raunio et al., 2008), including many nasal toxicants, such as the herbicide dichlobenil (Xie et al., 2010) and the antithyroid drug methimazole (Xie et al., 2011). Previous studies using recombinant P450
enzymes have shown that human CYP2A6 and CYP2A13, orthologs of mouse CYP2A5, are both active in NA bioactivation (Cho et al., 2006; Fukami et al., 2008); however, evidence for a role of CYP2A5 in mediating the toxicity of NA has yet to be obtained. To test our hypothesis, we compared NA metabolism and toxicity in Cyp2a5-null mouse, with germline deletion of the Cyp2a5 gene (Zhou et al., 2010), and wild-type (WT) mice. The Cyp2a5-null mouse model has been used in previous studies to demonstrate roles of CYP2A5 in the bioactivation of, and/or tissue toxicity induced by, several other compounds, including dichlobenil (Xie et al., 2010; 2013), acetaminophen (Zhou et al., 2011), methimazole (Xie et al. 2011), 3-methylindole (Zhou et al., 2012a), and the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Zhou et al., 2012b).

Here, the Cyp2a5-null and WT mice, both on the C57BL/6 (B6) genetic background, were treated with NA at a dose established in previous studies to induce respiratory tract toxicity in WT mice. The extent of NA-induced OM and lung toxicity was assessed by histological analysis, and through measurements of tissue levels of non-protein thiol (NPSH). The impacts of the loss of CYP2A5 expression on microsomal rates of NA metabolic activation in vitro, and on the kinetics of NA clearance in vivo, were also examined. Our results provide definitive evidence that CYP2A5 plays a critical role in mediating NA toxicity in the mouse OM.
Materials and Methods

Chemicals and animal treatments. Sources of NA (99% pure), NA-ds (99% pure), corn oil (highly refined, low acidity), GSH, NADPH, acetaminophen-glutathione (AP-GSH), NA-GSH standard (consisting of a mixture of all four stereoisomers), and all solvents (dichloromethane, formic acid, methanol, and water, all analytical grade) were the same as described recently (Li et al., 2011). Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. Cyp2a5-null and WT mice were obtained from breeding stocks maintained at the Wadsworth Center. Two- to three-month old, male mice were treated with a single dose of NA (200 mg/kg, i.p.) in corn oil. Control mice were injected with the vehicle only. Blood samples were collected from the tail at various times after dosing (15 min to 24 h) for preparation of plasma. At 24 h after NA injection, the mice were euthanized by CO2 overdose, and noses, lungs and livers were dissected for histopathological examination. For determination of tissue levels of NA-GSH and NPSH, liver, lung and OM were dissected at 2 h following NA injection. Plasma and tissue samples were stored at –80 °C until use.

Determination of catalytic activity in vitro. Microsomal NA metabolism was assayed as described recently (Li et al., 2011), by measuring rates of formation of NA-GSH (Shultz et al., 1999). Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, 2.5~200 μM NA (added in 2 μl of methanol), 10 mM GSH, 0.2 mg/ml (for lung) or 0.05 mg/ml (for OM) microsomal protein, and 1 mM NADPH, in a final volume of 0.2 ml. The reaction was carried out at 37 °C for 5 min. AP-GSH was added as internal standard. NA-GSH was determined using liquid chromatography-mass spectrometry (LC-MS) (Li et al., 2011). For controls, the reaction was terminated prior to NADPH addition. Apparent Km and Vmax values for OM microsomal formation of NA-GSH were estimated by nonlinear regression to Michaelis-Menten equation and the data reported as means ± S.D. of values determined for three separate microsomal samples, each prepared from tissues pooled from five mice.

Determination of NA, NA-GSH, and NPSH. Plasma NA levels were determined using GC-MS, as described (Li et al. 2011), with NA-ds as an internal standard. All procedures were carried out in sealed
tubes to prevent NA evaporation. Calibration curves were constructed using authentic NA (0.02–20 μg/ml), added to blank mouse plasma. NA-GSH levels in plasma and tissue homogenate samples from individual mice were determined as described (Li et al., 2011), using LC-MS/MS, with AP-GSH as an internal standard. Calibration curves were constructed using authentic NA-GSH (0.05–10 μg/ml), added to blank mouse plasma.

NPSH was determined as described (Xie et al., 2010), using an established method (Tonge et al., 1998). Liver and lung were homogenized with a Polytron (model GT 10-35, Kinematica, Bohemia, NY); whereas OM from individual mice was homogenized with use of a Bullet Blender (Next Advance, Averill Park, NY) (Xie et al., 2010). Reduced glutathione was used as the standard.

**Histopathological examination.** Liver and lung tissues were fixed in 10% neutral buffered formalin; whereas the nose was fixed in Bouin’s solution. Paraffin sections (4 μm) were stained with hematoxylin and eosin, for histopathological analysis (Gu et al., 2003, 2005). Nose tissue blocks were prepared according to Young (1981). The severity of lesions in the OM was graded, as described (Gu et al., 2005). Tissue sectioning and staining were performed at the Wadsworth Center Pathology core facility. Images were obtained using a Nikon model 50i light microscope, fitted with a digital camera, at the Wadsworth Center Light Microscopy core.

**Other methods.** Lung and OM microsomes were prepared as described previously (Gu et al., 1997). Pharmacokinetic parameters were calculated by non-compartmental pharmacokinetic analysis using the WinNonlin software (Pharsight, Mountain View, CA). Enzyme kinetic parameters were calculated using Prism (GraphPad Software, San Diego, CA). Statistical significance of differences between two groups in various parameters was examined using Student’s t test.
RESULTS and DISCUSSION

Role of CYP2A5 in OM Microsomal NA Metabolic Activation in vitro. The rates of NA-GSH formation in incubations of NA with OM or lung microsomes were compared between Cyp2a5-null and WT mice, with NA at 100 μM. OM rates were significantly higher in WT mice (0.62 ± 0.04 nmol/min/mg protein; mean ± SD, n=3) than in Cyp2a5-null mice (0.38 ± 0.01 nmol/min/mg protein; n=3); whereas lung rates were similar between the two strains (1.0 ± 0.1 and 1.1 ± 0.1 nmol/min/mg protein, respectively, for WT and Cyp2a5-null; n=3). OM microsomal activity was then further analyzed for determination of apparent kinetic constants. As shown in Figure 1, OM rates were consistently higher in WT than in Cyp2a5-null mice, over a broad range of NA concentrations. The apparent Vmax value was significantly higher in WT (0.74±0.01 nmol/min/mg protein; mean ± SD, n=3) than in the null mice (0.41±0.01 nmol/min/mg protein; P<0.01; compared to WT); whereas the apparent Km values were only slightly greater in WT (13.7±1.0 μM) than in the Cyp2a5-null mice (11.0±1.1 μM; P<0.05). The apparent catalytic efficiency (Vmax/Km; ml/min/mg protein) was 0.054 in WT OM and 0.037 in Cyp2a5-null OM. Thus, CYP2A5 plays an important role in NA bioactivation in the OM, and it seems to have (~50%) greater catalytic efficiency toward NA than the other P450 enzymes in OM microsomes.

Role of CYP2A5 in NA Metabolism in vivo. The loss of CYP2A5 in the Cyp2a5-null mice did not impact systemic elimination of NA or its major GSH-trapped metabolite NA-GSH. The pharmacokinetic profiles of plasma NA or NA-GSH were similar between WT and Cyp2a5-null mice, treated with NA at 200 mg/kg (i.p.) (Fig. S1 and Table 1). Although the Cyp2a5-null mice had slightly lower Tmax for plasma NA than WT mice had, there was no significant difference in t1/2, Cmax, AUC, or CL/F values, for either NA or NA-GSH. Thus, the loss of CYP2A5 expression did not alter systemic clearance of NA administered intraperitoneally.

Tissue levels of NA-GSH were also determined, in OM, lung, and liver, at 2 h after the NA treatment, in order to determine role of CYP2A5 in local NA metabolic activation in vivo. As shown in Table 2, the loss of CYP2A5 was associated with a 50% decrease in NA-GSH levels (compared with WT.
Role of CYP2A5 in NA-Induced Nasal Toxicity. NA is known to cause injury to the lung and OM regardless of the route of administration (Buckpitt et al., 2002). To determine whether CYP2A5 plays any role in NA toxicity, we compared the extent of tissue injury in WT and Cyp2a5-null mice treated with a known toxic NA dose (200 mg/kg i.p.; Plopper et al., 1992), at 24 h after dosing (Fig. 2). Obvious pathological changes were observed in NA-treated WT mice, in both olfactory epithelium and submucosa, especially in the nasal septum. Cells of the submucosal glands displayed features of coagulative necrosis and nuclear disintegration. The lesions in the epithelium included deformation and detachment. Remarkably, no lesion was observed in any of the NA-treated Cyp2a5-null mice examined. This result strongly supports the hypothesis that CYP2A5 plays a critical role in mediating the cytotoxicity of NA in the OM.

Signs of tissue damage, including necrosis and detachment of both Clara cells and ciliated airway epithelial cells, were also detected in the lungs of NA-treated mice (Fig. 2). However, in contrast to the situation for OM, and consistent with the absence of a significant impact of CYP2A5 loss on lung microsomal activity toward NA, there was no noticeable difference in the severity of lung toxicity between the Cyp2a5-null and WT mice. No obvious hepatotoxicity was observed in either WT or Cyp2a5-null mice at the dose studied (data not shown).

Levels of tissue NPSH, a marker of reactive metabolite formation in vivo (Phimister et al., 2004), were also determined for WT and Cyp2a5-null mice, at 2 h after NA dosing at 200 mg/kg (Table 2). For the liver, NPSH levels decreased by ~75% in WT mice and ~51% in Cyp2a5-null mice after the NA treatment, compared with corresponding vehicle-treated mice, a result indicating a relatively minor role of CYP2A5 in hepatic NA metabolic activation. Notably, the residual hepatic NPSH levels were still high in NA-treated mice, presumably explaining the absence of hepatotoxicity. For the lung, NPSH levels decreased by ~40% in both mouse strains after the NA treatment, consistent with the histopathological data showing that CYP2A5 does not play a significant role in NA-induced pneumotoxicity. For the OM,
NPSH levels decreased by ~94% in WT mice, but only ~44% in Cyp2a5-null mice, after the NA treatment, compared with corresponding vehicle-treated mice. The residual NPSH levels in the OM was ~10 times greater in NA-treated Cyp2a5-null mice, compared with NA-treated WT mice, consistent with the absence of nasal tissue injury in this group at 24 h after NA treatment.

Respective Roles of CYP2A5 and CYP2F2 in NA-Induced Respiratory Toxicity. The tissue difference in the impact of CYP2A5 loss on NA induced toxicity was consistent with in vitro metabolic data, which showed an obvious decrease in OM, but little change in lung, in rates of microsomal NA bioactivation. Similarly, the tissue difference in NA toxicity was consistent with in vivo biomarker data, which showed that the levels of NA-GSH, a marker for reactive NA metabolites produced in vivo, were significantly decreased only in OM, but not in lung or liver. On the contrary, rates of NA clearance were similar between Cyp2a5-null mouse and WT mouse, a fact indicating that the strain difference in OM toxicity did not result from a change in NA bioavailability in the Cyp2a5-null mice. The latter data also indicated that CYP2A5 did not make a notable contribution to circulating levels of NA-GSH, at least at the dose examined; this is consistent with our earlier data from Cyp2f2-null mice that CYP2F2 plays a predominant role in the systemic clearance of NA (Li et al., 2011).

NA was administered intraperitoneally in this and most other studies on mechanisms of NA toxicity. The respective roles of CYP2A5 and CYP2F2 in NA’s nasal and lung toxicities will unlikely change for NA exposure through other routes, such as inhalation, given the common location of the OM and lung in the respiratory tract. Furthermore, the tissue-selective contributions of the two enzymes to NA toxicity, which are at least partly determined by their relative abundances in OM and lung, will likely hold true for a broad range of NA doses, even though only a single dose was examined in the present study, as implied from the in vitro activity data for the Cyp2a5-null (here) and Cyp2f2-null (Li et al., 2011) mice. Notably, a detailed biochemical comparison of the NA metabolism by CYP2A5 and CYP2F2 in a reconstituted system remains to be conducted, in order to determine the relative catalytic efficiencies of the two enzymes, even though previous microsomal analysis showed that the apparent catalytic
efficiencies for NA bioactivation in lung (catalyzed mainly by CYP2F2) and OM (catalyzed mainly by CYP2A5) of WT mice, were comparable (Li et al., 2011).

**Summary.** We showed for the first time that mouse CYP2A5 plays an essential role in the bioactivation and toxicity of NA in the OM, but not in the lung. Our findings validate the respective roles of CYP2A5 and CYP2F2 in NA-induced nasal and pulmonary toxicity in mice, and illustrate the complexity of the metabolic mechanisms underlying site-specific toxicity of xenobiotic compounds in the respiratory tract, which dictates the necessity to study toxicant metabolism in each target tissue. These findings also provide a basis for predicting the toxic consequences of NA metabolism by human CYP2A6, CYP2A13, and CYP2F1 in the lung and nasal mucosa of exposed individuals.

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**Authorship Contributions**

*Participated in research design:* Hu, Sheng, L. Li, Zhou, Xie, D’Agostino, Y. Li, and Ding

*Conducted experiments:* Hu, Sheng, L. Li, Zhou, and Xie

*Contributed new reagents or analytic tools:* None

*Performed data analysis:* Hu, Sheng, L. Li, and Ding

*Wrote or contributed to the writing of the manuscript:* Hu, L. Li, Y. Li, and Ding
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Gu J, Zhang QY, Genter MB, Lipinskas TW, Negishi M, Nebert DW, and Ding X, 1998. Purification and characterization of heterologously expressed mouse CYP2A5 and CYP2G1: role in metabolic


Footnotes

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Legends to Figures

Fig. 1. Substrate-velocity curves for in vitro NA-GSH formation in OM microsomes. Contents of reaction mixtures are described under Materials and Methods. Microsomes were prepared from pooled OM from five 2- to 3-month old male mice. The rates of NA-GSH formation were determined at various NA concentrations (2.5 to 200 μM). The data (means ± S.D., n=3) were curve-fitted to the Michaelis-Menten equation.

Fig. 2. Histopathological analysis of nasal mucosal injury caused by NA treatment in WT and Cyp2a5-null mice. Two- to three-month old male mice were treated with a single i.p. injection of NA (at 200 mg/kg), or with the vehicle alone. Tissues were obtained for histopathological examination at 24 h after the treatment. For the nose, representative H&E-stained cross sections of the nasal cavity (40X), obtained at the level 3 of Young et al. (1981), are shown. Vehicle-treated mice had normal ethmoidal turbinates (ET) and nasal septum (SP) lined by a thick, pseudostratified olfactory neuroepithelium in the dorsomedial part, and a clear nasal passage; NA-treated WT B6 mice (n=6) showed extensive injury to the OM, evident as epithelial necrosis, detachment, sloughing, and ulceration; NA-treated Cyp2a5-null mice (n=5) showed essentially normal olfactory epithelium and Bowman’s glands (BG). For the lung, representative H&E-stained sections (40X) are shown. Control mice had a normal bronchiolar epithelium; NA-treated WT mice (n=6) and Cyp2a5-null mice (n=5) both showed necrosis and detachment of cells from the epithelium, with abundant cell debris (D) present in the airway lumen.
TABLE 1

Pharmacokinetic parameters for plasma NA and NA-GSH in mice treated with NA at 200 mg/kg

Two- to three-month old, male, WT and Cyp2a5-null mice were treated intraperitoneally with NA (at 200 mg/kg). Tail blood samples were collected from individual mice at various time points after dosing, for determination of plasma concentrations of NA and NA-GSH, which were used to calculate pharmacokinetic parameters. Values represent means ± S.D. (n = 5-6 for each strain). The plasma concentration-time curves are shown in Supplemental Figure 1.

<table>
<thead>
<tr>
<th>Analyte &amp; Strain</th>
<th>$t_{1/2}$ (h)</th>
<th>Tmax (h)</th>
<th>Cmax (μg/ml)</th>
<th>AUC$_{0-8h}$ (μg*h/ml)</th>
<th>CL/F (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.0±0.4</td>
<td>0.5±0.1</td>
<td>2.2±0.3</td>
<td>5.0±1.6</td>
<td>940±290</td>
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<tr>
<td>Cyp2a5-null</td>
<td>0.8±0.1</td>
<td>0.3±0.1$^a$</td>
<td>2.7±0.2</td>
<td>5.8±1.6</td>
<td>800±210</td>
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<tr>
<td>NA-GSH</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.8±0.3</td>
<td>2.0±0</td>
<td>4.4±0.8</td>
<td>12.6±3.3</td>
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<tr>
<td>Cyp2a5-null</td>
<td>0.7±0.1</td>
<td>1.4±0.8</td>
<td>3.9±1.5</td>
<td>10.6±4.1</td>
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</tbody>
</table>

$^aP < 0.01$, Student’s $t$-test, compared with corresponding WT values
Two- to three-month old, male, WT and Cyp2a5-null mice were given a single intraperitoneal injection of either vehicle alone or NA at 200 mg/kg. Tissues were collected from individual mice at 2 h after dosing. The data represent means ± S.D. (n = 3~6). Numbers in parentheses: null/WT (%).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Strain</th>
<th>NA-GSH (μg/g tissue)</th>
<th>NPSH (μmol/g tissue)</th>
<th>NA-induced decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>WT</td>
<td>53.6±7.2</td>
<td>8.4 ± 3.1</td>
<td>2.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Cyp2a5-null</td>
<td>49.8±6.6 (93%)</td>
<td>6.7 ± 1.6</td>
<td>3.3± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung</td>
<td>WT</td>
<td>5.4±1.4</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cyp2a5-null</td>
<td>4.5±1.1 (83%)</td>
<td>1.4 ± 0.3</td>
<td>0.9 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>OM</td>
<td>WT</td>
<td>1.8±0.4</td>
<td>1.8 ±0.1</td>
<td>0.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cyp2a5-null</td>
<td>0.9±0.5 (50%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8 ±0.1</td>
<td>1.0 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.01, NA treated compared with corresponding vehicle control values

<sup>b</sup>P < 0.01, Cyp2a5-null compared with corresponding WT values
Fig. 1

The graph shows the rate of NA-GSH formation (nmol/min/mg protein) as a function of NA concentration (μM) for WT and Cyp2a-null mice. The rate increases with increasing NA concentration for both genotypes, but the Cyp2a-null genotype has a lower rate compared to WT at all concentrations tested.
Fig. 2

WT Vehicle control

WT 200 mg/kg NA

Cyp2a5-null 200 mg/kg NA

OM

Lung

BG

ET

SP

D